Our Reference No. 7685-015

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
Wilfred A. Jefferies, Reinhard Gabathuler, Gerassimos Kolaitis and Gregor S.D. Reid	)
Serial No. **08/817,731**	) Group No.: **1632**
Filed: **July 21, 1997**	) Examiner: **Anne Marie S. Beckerleg**
For: **Method of Enhancing Expression of MHC Class I Molecules Bearing Endogenous Peptides**	)

The Commissioner of Patents
& Trademarks
Washington, D.C. 20231
U.S.A.

Dear Sir:

DECLARATION UNDER 37 CFR §1.132

I, Wilfred A. Jefferies, a citizen of Canada, and resident of South Surrey, British Columbia declare that the following facts are within my knowledge and are true.

1. I reside at 12596 23rd Avenue, South Surrey, British Columbia, Canada.

2. I am currently an Associate Professor at the University of British Columbia in the department of Biotechnology.

3. I have been conducting research in the field of Immunology since 1981. I have authored over 40 publications which have been published in refereed journals including Nature. I am an inventor on 3 issued or pending United States Patents. My curriculum vitae is attached to this Declaration as Exhibit A.

4. I am one of the inventors of U.S. Patent Application Serial No. 08/817,731 filed July 21, 1997 (hereinafter "the Application"). I have read and understood the disclosure and the claims of the Application.

5. I have read and understood the office action that issued on the Application on September 14, 1998. I understand that the Examiner has objected to the claims stating that the specification is only enabling for a method of enhancing *in vitro* the expression of MHC class I molecules bound to endogenous VSV peptides. Experiments have been conducted by me or under my direct supervision which demonstrate that the method of the invention can be used to enhance MHC class I molecule expression bound to peptides in addition to VSV and *in vivo*. Details of the experimental results are discussed in turn below.

6. **Other Peptides.**

In addition to the VSV peptides exemplified in the application, we have also demonstrated that introducing TAP-1 or TAP-2 into a cell also enhances the processing, presentation and expression of other viral antigens, tumor antigens and alloantigens.

(i) Tumor Antigens. This experiment demonstrates that introducing TAP-1 into a TAP deficient tumor enables the tumor to present a tumor associated antigen. As described in the Application, CMT.64 tumor cells are TAP-1 and TAP-2 deficient and when injected into mice cause solid tumors and death. (Examples 13 and 17 and Table 2). These examples also demonstrate that when the CMT.64 cells are transfected with both TAP-1 and TAP-2 the mice survive and exhibit no significant pathology. In the present experiment, CMT.64 cells were transfected with TAP-1 only to generate CMT1.4 cells. As a control, CMT.64 cells were transfected with a vector only to generate CMT.neo cells. We investigated whether CMT1.4 was presenting a tumor associated antigen that CMT.64 was not. A Cytotoxic T lymphocyte (CTL) analysis was performed using splenocytes from mice which had been injected with CMT.neo or CMT1.4. The experimental details and results are shown in

Figure 1 attached to the Declaration as Exhibit B. As can be seen from Figure 1, splenocytes from mice injected with CMT.neo recognized CMT.neo and CMT1.4 targets equivalently. Splenocytes from mice injected with CMT1.4 recognize CMT1.4 better than CMT.64. These results demonstrate that CMT1.4 presents a tumor associated antigen from CMT.64 which is being recognized by the host immune system but is not being presented by CMT.64.

(ii) Alloantigens. These experiments demonstrate that introducing TAP-1 or TAP-2 into a TAP deficient cell enables the cell to present allopeptides (alloantigens). In addition to the CMT.64 and CMT 1.4 cells described under (i) above, this experiment also employed CMT2.1 cells (transfected with TAP-2) and CMT12.12 cells (transfected with both TAP-1 and TAP-2). All of the CMT cells are H-2^b haplotype. Anti H-2^b alloreactive CTL cells were prepared by immunizing BALB/c mice (H-2^b) with spleen cells from C57/b6 (H-2^b) mice. We tested the ability of the anti H-2^b alloreactive CTLs to lyse CMT.64 and the CMT TAP transfectant cell lines CMT1.4, CMT2.1 and CMT12.12. As is shown in Figure 2 attached to the Declaration as Exhibit C, the alloreactive CTL cells recognized all of the CMT TAP transfectant cell lines at levels above the parental CMT.64 cells. These data demonstrate that TAP-1 or TAP-2 transfected cells are capable of transporting and presenting allopeptides.

In a second experiment, BALB/c mice were immunized and boosted 10 days later with CMT.64, CMT1.4, CMT2.10 or CMT12.12 cell lines. Alloreactive CTL were generated by limiting dilution analysis (LDA) and then tested against the CMT transfectant cells. The results are summarized in Table 1 which is attached to the Declaration as Exhibit D. As can be seen in Table 1, none of the alloreactive CTLs were able to lyse the untransfected CMT.64 cell line. These results further demonstrate that transfection of the cells with either TAP-1 or TAP-2 allows the processing and presentation of alloantigens.

(iii) Viral Antigens. These experiments demonstrate that transfecting cells with TAP-1 or TAP-2 enables processing and presentation of the Influenza A

antigen. The results are shown in Figure 3 which is attached to the Declaration as Exhibit E. The results demonstrate that CMT.64 cells transfected with TAP-1 (CMTr1) or TAP-2 (CMTr2) are able to present Influenza A antigen to Influenza Virus specific CTLs while CMT.64 cells, which are TAP deficient, can not.

7. ***In vivo* Data.** In order to demonstrate that the TAP molecules may function when administered *in vivo* the following experiment was conducted. $5x10^5$ CMT.neo cells (described above) were injected into 3 groups of mice. While one group received no treatment the other two groups were injected with either VV-rTAP1 or VV-pJS5 (a vector only control) 24 hours later and again at 2 weeks. The results and further experimental details are shown in Figure 4 which is attached to the Declaration as Exhibit F. As expected, the vector only control, VV-pJS5, ($p=0.18$) did not demonstrate any improvement over the group of mice injected with CMT.neo. However, the mice receiving VV-rTAP1 survived longer than the CMT.neo group ($p=0.01$), and the VV-pJS5 treated mice ($p=0.04$).

8. In view of all of the above, I believe that the claims as currently on file in the application are sufficiently enabled under 35 USC §112, first paragraph.

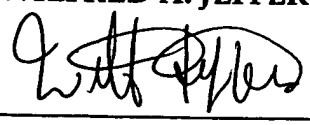
9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statement and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the Application or patent resulting therefrom.

4/23/99

DATE

Wilfred A. Jefferies

THIS IS **EXHIBIT "A"** MENTIONED AND REFERRED TO IN THE DECLARATION OF **WILFRED A. JEFFERIES**



Wilfred A. Jefferies

4/3/99

Date

THE UNIVERSITY OF BRITISH COLUMBIA
Curriculum Vitae for Faculty Members

Date: December 12, 1998 **Initials:** WJ

1. **SURNAME:** JEFFERIES **FIRST NAME:** Wilfred
MIDDLE NAME(S): Arthur

2. **DEPARTMENT/SCHOOL:** Biotechnology, Biomedical Research Centre, Medical Genetics, Zoology Microbiology and Immunology

3. **FACULTY:** Medicine, Science

4. **PRESENT RANK:** Associate Professor **SINCE:** July 1994

5. **POST-SECONDARY EDUCATION**

University or Institution	Degree	Subject Area	Dates
University of Victoria	B.Sc.	Biochemistry (First Class with Distinction)	1981
University of Oxford	D. Phil. (Oxon)	Molecular Immunology	1985

Special Professional Qualifications

6. **EMPLOYMENT RECORD**

(a) Prior to coming to UBC

University, Company or Organization	Rank or Title	Dates
Department of Biochemistry and Microbiology, University of Victoria	Undergraduate Research Assistant	1979
Terry Fox Lab, B.C. Cancer Control Agency, UBC	Undergraduate Research Project	1980
Dept. of Pediatrics, UBC	Work-Studies Student	1981
Institut Suisse de Recherches Expérimentales sur le Cancer, Lausanne, Suisse	Postdoctoral Research Fellow	1985-1987
Ludwig Institute for Cancer Research, Karolinska Institute, Stockholm, Sweden	Postdoctoral Research Fellow	1987-1989

(b) At UBC

Rank or Title	date
Assistant Professor	1989-1994
Associate Professor	1994-present

(c) Date of granting of tenure at U.B.C.: July 1, 1994

7. LEAVES OF ABSENCE

University, Company or Organization at which Leave was taken	Type of Leave	Dates

8. TEACHING

(a) Areas of special interest and accomplishments

(b) Courses Taught at UBC

Session	Course Number	Scheduled Hours	Class Size	Hours Taught			
				Lectures	Tutorials	Labs	Other
1989-90	MICRO 530	36	12	6			
	BIOL 350	36	150	2			
	BIOL 437		2			>100	Research projects
	BIOL 448		2			"	
	BIOL 449		1			"	
1990-91	MICRO 430	36	20	6		>100	Research projects
	BIOL 437		2				
90-91 (summer)	MICRO 200	72	100	15			
	BIOL 448		2			"	
	MICRO402/ MED GENET 410	36	40	13			
	MICRO 302	36	150	20			
	PHARMAC 521	36	15	1			
1991-92	BIOL 437		2				
	BIOL 448		2			>100	Research projects
	MICRO 200	72	275	13		"	
	MICRO 402/ MED GENET 410 (Course Organizer)	36	100	13			
1992-93	MICRO 200	72	275	13			
	MICRO 530	36	12	6			
	BIOL 437		2			>100	Research projects
	BIOL 448		2			"	
	MICRO 402/ MED GENET 410 (Course Organizer)		100	13			

1993-94	MICRO530 BIOL 437 BIOL 448 MICRO 200 MICRO 402/MED GENET 410 (Course Organizer)	72 72 36	12 2 2 275 100	6 13 13		>100 "	Research Projects
1994-95	BIOL 437 BIOL 448 MICRO 200 MICRO 402/ MED GENET 410 (Course Organizer)	 72 36	2 2 275 100	 13 13		>100 "	Research Projects
1995-96	BIOL 448 MICRO 202 MICRO 402/ MED GENET 410 (Course Organizer)	 36 36	3 275 70	 15 13	1	>100	Research Projects
1996-97	BIOL 448 MICRO 202 MICRO 402/ MED GENET 410 (Course Organizer)	 36 36	3 275 70	 15 13	1	>100	Research Projects
1997-98	BIOL 448 MICRO 202 - Section 1 Section 3 MICRO 402/ MED GENET 410 (Course Organizer)	 36 36 36	3 275 275 70	 12 12 13	 1 1	>100	Research Projects

c) Graduate Students Supervised

Student Name	Program Type	Year		Principal Supervisor	Co-Supervisor(s)
		Start	Finish		
Mike Food	M.Sc.		1992	W. Jefferies	
Kathy Shimizu	M.Sc.		1994	"	
Roger Lippé	Ph.D.		1995	"	
Ian Haidl	Ph.D.		1996	"	
Gregor Reid	Ph.D.		1996	"	
Cyprien Lomas	Ph.D.		Underway	"	
Joseph Yang	M.Sc.		Underway	"	
Judie Alimonti	Ph.D.		1998	"	
Greg Lizee	Ph.D.		Underway	"	
Forrest Hsu	M.Sc.		1997	"	
Jacqueline Tiong	Ph.D.		Underway	"	
Alex Moise	Ph.D.		Underway	"	
Michael Bromm	M.Sc.		Underway	"	
Dara Dickstein	M.Sc.		Underway	"	

Maya Kotturi	M.Sc.		Underway	"	
Brandie Walker	Ph.D.		Underway	"	

Post-doctoral Fellows Supervised:

		Current Position
1991-1992	Jonas Ekstrand (Swedish Research Council Fellowship)	Assistant Professor Emory University, Atlanta,U.S.A.
1991-1993	Sylvia Rothenberger (Swiss National Research Foundation)	Research Associate Lausanne, Switzerland
1994-1996	Catherine Barbey (Swiss National Research Foundation)	Post-doctoral Researcher, Jefferies' Lab

Research Associates:

1991-	Reinhard Gabathuler (Tobacco Research Council)
1993-94	Chris Nichol (Synapse Technologies Inc.)
1993-	Malcom Kennard (Synapse Technologies Inc.)

(d) Continuing Education Activities

(e) Visiting Lecturer (indicate university/organization and dates)

37. Visiting Scientist

1998-	Simon Hunt, Ph.D.	Sabbatical Visitor University of Oxford, U.K.

(g) Other

Thesis Committee member for: (21)	Supervisor
Bruce Banfield; Peter Cheung	Frank Tufaro
Kathy Horley; Andrew Pyszniuk	Fumio Takei
Clay Welder, Andrea Ingram, Carmine Carpenito, Mary Gilbert	Don Moerman
Richard Hegele	Jim Hogg
William Craig	Connie Eaves/Peter Lansdorp
Stephen Land	Peter Hochachka
David Hunt	Julia Levy
Chris Fraser	Keith Humphries
Patrick Rebstein	Gerry Weeks
Arpeta Matia	Pauline Johnson
Bevan Voth, Jaime Bellatin	Rob McMaster
Amanda Jones	Hermann Ziltner
Bevan Voth, Jaime Bellatin	Rob McMaster
Amanda Jones	Hermann Ziltner
Jose Rey-Ladino	Niel Reiner
John Chiu	Alan Eaves
Madelaine Lemieux	Connie Eaves

Dissertation Committee member for: (11)	
Chris Fraser, Ph.D.	Keith Humphries

Mark Daly, MSc.	Hugh Brock
Carmine Carpenito, MSc	Fumio Takei
Peter Cheung, MSc.	Frank Tufaro
Mike Food, M.Sc.	Wilfred Jefferies
Clay Welder, M.Sc.	Fumio Takei
Richard Hegele, Ph. D.	Jim Hogg
William Craig, M.Sc.	Connie Eaves/Peter Lansdorp
Helena Chaye, Ph.D.	Shirley Gillam
Mark Daly, MSc.	Hugh Brock
Carmine Carpenito, MSc	Fumio Takei
Brad Spiller, Ph. D.	Dana Devine
Mary MacDonald Ph.D.	Rob McMaster
Charllotte Morrison Ph.D.	Ron McMaster
Andrew Pyszniuk Ph.D.	Fumio Takei
External examiner	
Mark Luscher Ph. D. Toronto	Brian Barber

9. SCHOLARLY AND PROFESSIONAL ACTIVITIES

38. Areas of special interest and accomplishments
Antigen processing, Autoimmunity, Viral immunity, Iron metabolism, Alzheimer's Disease Biomarkers

Summary of Interests:

1. We work on the transport of metals into the brain and have cloned and identified components which appear to be involved in this process.

2. AD Diagnostic: Our AD diagnostic test and prognosticator for Alzheimer's Disease (AD) has gained wide interest from those in the field because it is a serum test and the concentration of the novel protein p97 we are measuring appears to increase with disease severity. Furthermore, the concentration increases prior to the onset of the symptoms of the disease and the biomarker may be useful in quantitatively assessing the outcome of clinical trials. At present there is no other indicator of AD which has these characteristics and therefore this test may fundamentally change clinical approaches in the treatment of AD. For example, overwhelming evidence in the field suggests that most of the neurons which die in patients with AD do so prior to the clinical manifestations of the symptoms of the disease. If one could identify individuals prior to their symptomatic presentation, then treatments may be more effective against this disease. Therefore, the test we have devised may have some important uses in detecting and treating AD.

3. Our lab has cloned and identified components of the system which degrades and transports foreign proteins for recognition by the immune response. We are at present manipulating these components to increase the immune response against viruses and tumours.

4. We have identified novel components of persistent viral infections in man and are currently analyzing these components in order to understand which aspects of the host immune response these viruses evade.

5. Technically, my lab provides a service for the custom preparation of rabbit antisera, virus production and recombinant expression systems. In the future my lab will provide transgenic animal technologies to the greater biotechnology community.

6. I serve as the Director of the new Centre for Molecular Immunology which is designed to promote the interactions amongst the B.C. Immunology community.

(b) Research or equivalent grants (indicate under COMP whether grants were obtained competitively (C) or non-competitively (NC))

Granting Agency	Subject	COMP	$ per year	Year	Principal Investigator	Co-Investigator(s)
National Cancer Institute of Canada	Molecular studies on the transport, recognition and inhibition of MHC Class I gene products	C	45,088 47,539 61,000	89-90 90-91 91-94	W. Jefferies	
National Cancer Institute of Canada	**Equipment** for above	C	36,610	89-91	"	
National Cancer Institute of Canada	Molecular studies on the transport, recognition and inhibition of MHC Class I gene products and characterization of the melanoma-associated protein, melanotransferrin	C	63,025 59,749 60,934	91-92 92-93 93-94	"	
National Cancer Institute of Canada	Viral and genetic mechanisms which lower MHC Class I cell surface expression	C	103,000	94-97	"	
British Columbia Health Research Foundation	The role of the E3/19k protein of Adenovirus-2 in lowering the cell surface expression of HLA molecules and in viral persistence	C	45,000 50,000	89-90 90-91	"	
British Columbia Health Research Foundation	The function of the melanoma associated protein melanotransferrin	C	27,000	92-94	"	
British Columbia Health Research Foundation	**Equipment** for Molecular action of cyclosporin A	C	14,000	89-90	"	
British Columbia Health Research Foundation Emergency	**Equipment** - Cryopreservation unit	C	5,000	91-92	"	
Natural Science and Engineering Council of Canada - UBC	**Equipment** for The generation of antigen presenting mutants	C	25,000	1990	"	
Natural Science and Engineering Council of Canada	**Equipment** - Cryopreservation unit	C	8,000	90-91	"	
American Council for Tobacco Research	The role of the E3/19k protein of Adenovirus-2 in lowering the cell surface expression of HLA molecules and in viral persistence	C	91,000 101,000 112,000 60,000 88,400 91,000	1991 1992 1993 1994 1995 1996	"	
Vancouver Foundation/Medical Services Association	An early detection test for Alzheimer's disease	C	45,000	91-93	"	
Medical Research Council of Canada	Human melanotransferrin	C	74,000	93-97	"	
Medical Research Council of Canada	Molecular characterization of antigen processing variants	C	60,000	93-97	"	

Synapse Technologies Inc	Melanotransferrin	C	125,000	97-2000	"	
B.C. Science Council	An early detection test for Alzheimer's disease	C	45,000 104,000	95-96 97-98	"	
National Centre of Excellence for Neural Regeneration	Transport across the Blood Brain Barrier	C	48,000	93-98	"	
Medical Research Council of Canada	Characterisation of the function of Melanotransferrin	C	52,673	97-2001	"	
Medical Research Council of Canada	Antigen Processing in the Context of MHC Class I molecules	C	94,359	97-2001	"	
Natural Science and Engineering Research Council of Canada	Novel eukaryotic expression systems	C	108,760	97-2001	"	
National Cancer Institute of Canada	Animal models of adenovirus persistence	C	25,356	97-98	"	
National Cancer Institute of Canada	Studies on the structure and function of MHC Class I molecules	C	25,356	97-98	"	

(c) *Research or equivalent contracts (indicate under COMP whether grants were obtained competitively (C) or non-competitively (NC).*

(d) *Key Recent Invited Presentations*

1997 Molecular Biology of Alzheimer's Disease,
San Francisco, California, p97 and Alzheimer's Disease

(e) *Other Presentations*
Newspaper interviews 1996-97

(f) *Other*

National Professional Committees

1991-92 National Cancer Institute of Canada fellowship, scholarship, and career award panel member.

1992-93 National Cancer Institute of Canada, Immunology Grants Committee

1993-94 National Cancer Institute of Canada, Immunology Grants Committee

(g) *Conference Participation (Organizer, Keynote Speaker, etc.)*

Conference Keynote Speaker

1988 The Joint Swedish-Israeli Workshop on Biophysical Interactions, Stockholm, Sweden -Studies on MHC Class I molecules.

1992	Canada Society for Immunology keynote speaker, Montreal, Quebec - The Adenovirus-2 E3/19K protein down regulates the host immune response.
1996	Convenor ASM Meeting, Newport Oregon Persistence mechanisms of Viruses

10. SERVICE TO THE UNIVERSITY

(a) Memberships on committees, including offices held and dates

1990-present:	Founder and Organizer of the Campus Wide Immunology Study Group
1990-present:	Member U.B.C. Biochemical Discussion Group
1990-present:	Member U.B.C. Immunology Seminar Group
1991:	Chairman, Department of Microbiology, Pathogenesis Course Evaluation Committee
1991:	Member, Department of Microbiology, Immunology Course Evaluation Committee
1991:	Member, Department of Microbiology, Cell Biology Course Evaluation Committee
1991:	Member, Department of Microbiology, Immunology Curriculum Committee
1992:	Medicine 2000 Immunology/Transplantation Organizing Committee
1992:	Secretary Biotechnology Safety Committee
1993:	Member, Adhoc Committee, Microbiology 200
1993:	Secretary Biotechnology Safety Committee
1993:	Member, Undergraduate Genetic courses Evaluation Committee, Medical Genetics
1995-present	Graduate Council Representative for the Department of MIcrobiology and Immunology
1996:	Chairman Biotechnology Laboratory Awards Committee
1997:	Faculty of Science committee for Dissertation Gold Medal
1998:	Search Committee, Biotechnology Laboratory, Instructor position
1998:	Tenure Committee, Microbiology and Immunology, Michael Gold
1998:	Search Committee, Microbiology and Immunology, Virology Position
1998:	Committee to review B.C. Biotechnology Alliance High School Research Applications
1998-99:	Organizing Campus Wide Transgenic Animal Facility
1998-99:	**Director**, U.B.C. Centre for Molecular Immunology

(b) Other service, including dates

1989: U.B.C. Program for Effective Teaching Methods -One day seminar
1991: U.B.C. Program for Effective Teaching Techniques-Three day seminar

11. SERVICE TO THE COMMUNITY

(a) Memberships on scholarly societies, including offices held and dates

1982- present	British Biochemical Society
1982-1989	British Transplantation Society
1982- present	British Society of Immunology
1990-present	Canadian Society of Immunology

(b) Memberships on other societies, including offices held and dates

(c) Memberships on scholarly committees, including offices held and dates
Member of the Ronald and Nancy Alzheimer's Research Association committee on Emerging Biomarkers for Alzheimer's Disease
The"Consensus Report of the Working Group on: 'Molecular and Biochemical Markers of Alzheimer's Disease,'" w appears in the April 1998 issue of the journal Neurobiology of Aging.

(d) Memberships on other committees, including offices held and dates
(e) Editorships (list journal and dates)

(f) Reviewer (journal, agency, etc. including dates)

Peer Reviews: Grants

1990 -	Natural Science and Engineering Research Council of Canada Operating grant
1990 -	Natural Science and Engineering Research Council of Canada Strategic grant
1991 -	Natural Science and Engineering Research Council of Canada Strategic grant
1991 -	Medical Research Council of Canada Operating grants
1991/92 -	Medical Research Council of Canada grants
1991 -	St. Paul's Hospital Fnd. grant
1992-	National Cancer Institute of Canada, Career Fellowships
1992-	Alberta Cancer Society grant
1992-93-	Medical Research Council of Canada Operating grants
1992-93-	National Cancer Institute of Canada Operating grants
1993-94-	Medical Research Council of Canada Operating grants
1993-94-	National Cancer Institute of Canada Operating grants
1994-	Alberta Cancer Society grant
1996	Medical Research Council of Canada Operating grants
1997	Medical Research Council of Canada Operating grants
1997-	Manitoba Research Foundation
1998	Medical Research Council of Canada Operating grants
1998-	Manitoba Research Foundation
1998-	Dalhousie Research Foundation
1998	Heart and Stoke Foundation

Peer Reviews: Journals
FEBS Letters
International Immunology
Biochem Biophys Acta
J. of Neuroscience
Blood
J. of Leukocyte Biology

National Grant Review Panels

1992-	National Cancer Institute of Canada Career Award and Fellowship Panel
1993-	National Cancer Institute of Canada Immunology Panel
1994-	National Cancer Institute of Canada Immunology Panel

(g) External examiner (indicate universities and dates)

Mark Luscher Ph. D. Toronto Brian Barber

(h) Consultant (indicate organization and dates)

Board of Directors, Scientific Director, Synapse Technologies Incorporated.

(i) Other service to the community

1990:	Host of the EUCLID recipients, Award winners of the province wide Math prizes
1990:	Host Organizer/Lecturer of U.B.C. Connect 90' B.C. High School Science Student Forum
1990:	Faculty Representative at the Convocation Ceremonies for the Faculty of Science
1990:	Faculty of Science, Open House Display
1991:	Lecturer: Three day seminar U.B.C. Connect 91' B.C. High School Science Student Forum
1991:	Judge for the Canada Wide Science Fair for Canadian High School Students

1991:	Faculty Representative at the U.B.C. Convocation Ceremonies for the Faculty of Science
1991:	OUTREACH Volunteer for U.B.C participation in High School Graduation Ceremonies
1992:	Faculty Representative at the U.B.C. Convocation Ceremonies for the Faculty of Science
1992:	University sponsored lecture: Shad Valley Program
1993:	Faculty Representative at the U.B.C. Convocation Ceremonies for the Faculty of Science
1993:	University sponsored lecture: Shad Valley Program for exceptional B.C. High school students
1993-Present:	Lecturer: Three day seminar U.B.C. ***Connect*** B.C. High School Science Student Forum
1998:	Supervised two High School Co-op Research Projects

12. AWARDS AND DISTINCTIONS

(a) Awards for Teaching (indicate name of award, awarding organizations, date)

(b) Awards for Scholarship (indicate name of award, awarding organizations, date)

1985	Lady Tata Memorial Leukemia Fellowship
1985	Royal Society of London Postdoctoral Fellowship
1985-86	Welcome Trust Postdoctoral Fellowship
1986-89	Medical Research Council of Canada Postdoctoral Fellowship
1990	Nominated for Hughes Institutes for Medical Research Centre
1994-	Named Principal Investigator for the National Network of Excellence for Neural Regeneration
1995	Nominated for Hughes Institute for Medical Research International Scholarship

(c) Awards for Service (indicate name of award, awarding organizations, date)

(d) Other Awards

Academic awards and distinctions (prior to final degree):

1980	British Columbia Cancer Research Summer Scholarship
1982-1985	Overseas Research Student Scholarship (British)
1982-84	British Columbia Health Care Trainingship Award
1982	Natural Science and Engineering Council of Canada Graduate Scholarship
1982-85	Royal Commission for Exhibition of 1851 Scholarship (London) (10 offered world-wide each year)

13. OTHER RELEVANT INFORMATION (Maximum One Page)

THE UNIVERSITY OF BRITISH COLUMBIA
Publications Record

Date: December 12th, 1998 **Initials:** _____

SURNAME: JEFFERIES **FIRST NAME:** Wilfred **MIDDLE NAME(S):** Arthur

	Summary of Refereed Publications		Summary of Non-Refereed Publications		
	1A *(journals)*	1B *(reviews)*	2 *(conf. pro.)*	3 *(books)*	4 *(patents)*
Career	37	1	34	4	4
Last 5 Years	20	1	16	3	3

Career Total: 80
Last Five Years: 43

1. REFEREED PUBLICATIONS

(a) Journals (denotes publications of primary importance)*

*1. ***Jefferies, W.A.***, Brandon, M.R., Hunt, S.V., Williams, A.F., Gatter, K.C., and Mason, D.W., (1984), Transferrin receptors on endothelium of brain capillaries. ***Nature*** 312:162-163.

2. ***Jefferies, W.A.***, Brandon, M.R., Williams, A.F., Hunt, S.V., (1985), A monoclonal antibody against the rat transferrin receptor does not detectably label most lymphopoietic stem cells. ***Immunology*** 54:333-341.

3. ***Jefferies, W.A.***, Green, J.R., and Williams, A.F. (1985), Authentic rat T helper cell antigen CD4(W3/25) expressed on peritoneal macrophages. ***J. Exp. Med.*** 162:117-127.

4. Arvieux, J., ***Jefferies, W.A.***, Paterson, D.J., Williams, A.F., and Green, J.R. (1986), Monoclonal antibodies against a rat leukocyte antigen which blocks antigen-induced T cell responses via an effect on accessory cells. ***Immunology*** 58:337-342.

*5. ***Jefferies, W.A.***, Barclay, A.N., Gagnon, J., and Williams, A.F., (1986), Structure of the CD4(W3/25) T-helper lymphocyte glycoprotein. ***Biochemical Society Transactions*** 14(2):336.

6. Paterson, D.J., Green, J.R., ***Jefferies, W.A.***, and Williams, A.F. (1986), Functional thymic populations in the rat as defined by the monoclonal antibody MRC OX44. ***J. Exp. Med.*** 165:1-13.

*7. Clark, S.J., ***Jefferies, W.A.***, Barclay, A.N. Gagnon, J., Williams, A.F. (1987), Peptide and nucleotide sequences of rat CD4(W3/25) antigen: Derivation from a structure with four immunoglobulin-related domains. ***Proc. Natl. Acad. Sci. U.S.A.*** 84:1649-1653.

8. Crocker, P.R., ***Jefferies, W.A.***, Clark, S.J., Chung, P.L., and Gordon, S., (1987), Species heterogeneity in macrophage expression of the CD4 antigen (AIDS virus receptor). ***J. Exp. Med.*** 166:613-618.

9. ***Jefferies, W.A.*** and MacPherson, G.G., (1987), Expression of the W6/32 HLA class I epitope by cells of rat, mouse, human and other species: Critical dependence on the interaction of bovine or human beta-2 microglobulin. ***Eur. J. of Immunol.***, 17:1257-1263.

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14 Matsuse, T., Hayashi, S., Kuwano, K., Keunecke, H., **Jefferies, W.A**. and Hogg, J.C. (1992).Latent adenoviral infection in the pathogenesis of chronic airways obstruction. ***Am. Rev. Respir Dis.*** 146: 177-184

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*21. Food, M., Rothenberger, S., Gabathuler, R., Haidl, I., Reid, G., and **Jefferies, W.A.** (1994) Transport and expression in human melanomas of a transferrin-like glycosylphosphatidylinositol anchored protein. ***J. Biol. Chem.*** 269: 3034-3040.

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25. Garcia-del Portillo, F., Pucciarelli, M.G., **Jefferies, W.A.**, and Finlay, B.B. (1994) Salmonella typhimurium induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. ***J. Cell Science*** 107: 2005-2020.

*26. Kennard, M., Richardson, D. Gabathuler, R., Ponka, P., **Jefferies, W.A.** (1995) A novel iron uptake mechanism mediated by GPI-anchored human p97. ***EMBO Journal*** 14: 4178-4186.

27. Haidl, I., Rothenberger, S., Johnson, P., **Jefferies, W.A.** (1995) Detection of restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. ***Eur. J. Immunol.*** 25: 3370-3374.

28. Rothenberger, S., Food, M.R., Gabathuler, R., Kennard, M., Yamada, T., Yasuhara, O., McGeer, P.L., **Jefferies, W.A.** (1996) Coincident expression and distribution of melanotransferrin and transferrin receptor in human brain capillary endothelium. ***Brain Research*** 712:117-121.

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35. Yamada, T., Yoshiyama, Y., Kawaguchi, N., Ichinose, A., Iwaki, T., Hirose, S., ***Jefferies , W.A.*** (1998) Possible role of transglutaminases in Alzheimer's disease. ***Dement Geriatr Cogn Disord, 103-110***

36. Gabathuler, R., Alimonti, J., Zhang, Q-J., Kolaitis, G., Reid, G., **Jefferies, W.A.** (1998) Surrogate antigen processing and the alternative fate of antigenic peptides within the secretory pathway. ***Journal of Cell Biology 140, 17-27.***

37. Hmama, Z., Gabathuler, R., ***Jefferies, W.A.***, De Jong, G. and Reiner, N.E. (1998) Attenuation of HLA-DR Expression by Mononuclear Phagocytes Infected with *Mycobacterium tuberculosis* Is Related to Intracellular Sequestration of Immature Class II Heterodimers. ***J. Immunol.*** 161:4882-4893.

b). Refereed Review

1. **Jefferies, W.A.**, Gabathuler, R., Rothenberger, S., Food, M., Kennard, M. (1996) <u>Pumping iron in the 90's</u>. ***Trends in Cell Biology*** 6:223-228.

2. NON-REFEREED PUBLICATIONS

(a) Journals

1. ***Jefferies, W.A.*** et al (1998) "Consensus Report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease,' ***Neurobiology of Aging*** 4, 1-20.

(b) Conference Proceedings

1. ***Jefferies, W.A.***, Brandon, M.R., Hunt, S.V., and Williams, A.F. Characterization of the rat transferrin receptor. Harden Conference on the Cell Surface Proteins of Lymphocytes, Kent, England, 1982.

2. ***Jefferies, W.A.***, Hunt, S.V., and Williams, A.F. Characterization of the rat receptor for transferrin. Harden Conference on Cell Surface Proteins, Wye College, 1982.

3. ***Jefferies, W.A.***, Brandon, M.R., Hunt, S.V., and Williams, A.F. A monoclonal antibody against rat transferrin receptor does not detectably label lymphopoietic stem cells. EMBO Workshop on T Lymphocyte Cloning, Marseille, France, 1983.

4. ***Jefferies, W.A.***, Barclay, A.N., Gagnon, J., and Williams, A.F. Structure of the CD4(W3/25) T-Helper Lymphocyte Glycoprotein, Biochemical Society, London, England, 1985.

5. ***Jefferies, W.A.***, Clark, S., Barclay, A.N., Gagnon, J., and Williams, A.F. Structure of the rat CD4(W3/25) glycoprotein of T helper lymphocytes. Sixth International Congress of Immunology, Toronto, Ontario, Canada, 1986.

6. ***Jefferies, W..A.***, and Kvist, S. Using antisense segments of the H-2 K^k to inhibit cell surface expression of MHC Class I proteins. H-2 and HLA Workshop, Montreaux, Switzerland, 1986.

7. Burgert, H-G., ***Jefferies, W.A*** and Kvist, S. The E3/19K protein of Adenovirus 2 binds to HLA molecules intracellular and inhibits recognition by cytolytic T lymphocytes, International Congress of Virology, Edmonton, Alberta, Canada, 1987.

8. ***Jefferies, W.A.***, Ruther, U., Wagner, E., Kvist, S. A Null MHC Class I Restriction Element Becomes Functions in Transgenic Mice, H-2 and HLA Workshop, Airlie House, Virginia, U.S.A., 1988.

9. ***Jefferies, W.A.***, and Kvist, S. Using antisense RNA in the inhibition of H-2 molecules. EMBO/INSERM Workshop on Antisense RNA, Savoie, France, 1988.

10. ***Jefferies, W.A.***, Ruther, U., Wagner, E., and Kvist, S. Cytolytic T cells recognize a chimeric MHC Class I antigen expressed in influenza A infected transgenic mice. The Swedish-Israeli Workshop on the molecular basis of biological recognition, membrane dynamics and transport. Sodergarn Mansion, Stockholm, 1988.

11. Burgert, H.-G., ***Jefferies, W.A.***, and Kvist, S. The role of the E3/19k protein from Adenovirus-2 in the inhibition of HLA expression. International Virus Congress, Berlin FRG, 1990.

12. Matsuse, T., Hayashi, S., Hogg, J.C., Keunnecke, H., ***Jefferies, W.A.*** Detection of Adenoviral early glycoprotein E3/19k gene in frozen lung tissue by the polymerase chain reaction (PCR). American Lung Association Meeting, Annhem, California, USA., 1991.

13. ***Jefferies, W.A.***, Kolaitis, G. and Gabathuler, R. A murine cell variant differentially presents antigens derived after VSV or Influenza infection. J. Cell Biochem., Supplement 16D, p. 15, Keystone Symposium, 1992.

14. Lomas, C., Lippé, R., ***W.A. Jefferies***. The adenovirus E3/19K protein blocks the phosphorylation of MHC molecules. IBID, p. 17.

15. Lippé, R., Kolaitis, G. and ***Jefferies, W.A.*** Differentiated processing of viral and allogeneic peptides. IBID, p. 50.

16. Haidl, I., Rothenberger, S., Johnson, P. and ***Jefferies., W.A.*** Characterization of the CD45 isoforms expressed by mouse dendritic cells. International Congress on Dentritic Cells, Netherlands, 1992.

17. Kennard, M., Rothenberger, S., Food, M., ***Jefferies, W.A.*** and Piret, J. A novel method for expression of mammalian glycoproteins. American Society of Molecular Engineers, 1992.

18. Lippé, R., Lomas C., and ***Jefferies, W.A.***, Phosphorylation of MHC takes place in a Post-ER compartment, Canadian Society of Microbiologists, Vancouver, B.C., November, 1991

19. Lippé, R., Lomas C., and ***Jefferies, W.A.***, Regulation of the Phosphorylation of MHC Class I molecules by the E3 region of Adenovirus Type 2, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.

20. Haidl, I and ***Jefferies, W.A.***, Molecular analysis of the F4/80 antigen, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.

21. Shimizu, K., Theilman, D., and ***Jefferies, W.A.*** Expression of recombinant forms of Immunophillins in Baculovirus, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.

22. Reid, G., Gabathuler, R., and ***Jefferies, W.A.*** TAP-1 is involved in peptide transport in the TAP-2-deficient RMA-S cell line. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

23. Lomas, Cyprien, Gabathuler, R., and ***Jefferies, W.A.*** The Adenovirus protein E3/19K exhibits chaperone-like behaviour in the endoplasmic reticulum. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

24. Barbey, C., Watts, C., and Corradin, G., and ***Jefferies, W.A.*** Antigen processing organelles from DRB1*1101 and DRB*1104 cell lines display a differential enzymatic activitiy. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

25. Gabathuler, R., Reid, Gregor, Kolaitis, Gerassimos, and ***Jefferies, W.A.*** A novel TAP transporter is peptide selective. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

26. Kennard, M.L., Tiong, J., and ***Jefferies, W.A.*** Screening hybridomas by fluorescence concentration analysis. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

27. Haidl, I.D., Ng, D., Johnson, P., and ***Jefferies, W.A.*** Restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

28. Lippé, R. and ***Jefferies, W.A.*** Characterization of novel adenoviral protein and its ability to modulate MHC class I surface expression. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.

29. ***Jefferies, W.A.*** Kennard, M.L., Gabathuler, R., Food, M. R., Yamada, T., McGeer, P. Studies on the melanotransferrin molecule. National Centre of Excellence, Neural regeneration Network, June, 1995, St. Adéle, Que.

30. Barbey, C., Gabathuler, R., Reid, G., Kolaitis, G., ***Jefferies, W.A.***, Determination of the TAP transporters' specificity in the viral processing and presentation deficient cell line CMT.64. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.

31. Gabathuler, R., Kennard, M., Food, M., Richardson, D., Ponka, P., Rothenberger, S., ***Jefferies, W.A.*** Characterisation of a GPI-linked protein, Melanotransferrin (p97), involved in transferrin-indepedent uptake of iron. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.

32. Reid, G., Gabathuler, G., Kolaitis, G., ***Jefferies, W.A.***, Tap-1 is sufficient for the transport of selected peptides. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.

33. Feldman H., Kennard, M., Yamada, T., Adams, S., ***Jefferies, W.A.*** Serum levels of the Iron Binding Protein p97: A novel biological marker of Alzheimer's Disease. Fifth International conference Disease and related disorders, Osaka Japan, 24th July, 1996

34. Hegedus, D., Pfeifer, T., Kennard, M., Gabathuler, R., ***Jefferies, W.A.***, Theilmann, D. and Grigliatti, T (1997). Stable insect cell line based protein expression. Annual scientific meeting of Canadian Insect Biotechnology, Toronto, Ontario June 1997.

35. ***W.A. Jefferies***, H.Feldman., J. Nurminen, D. Levy, D. Foti, S. Foti, M. Kennard, and R. Gabathuler Serum p97 levels as a sscreening test for Alzheimer's disease.
6th International conference on Alzheimer's Disease and Related Disorders Amstersdam, The Netherlands 18-23 July, 1998

(c) Other

3. BOOKS

(a) Authored

(b) Edited

(c) Chapters

*1. ***Jefferies, W.A.*** (1988). Hemopoietic and T Lymphocyte Marker Antigens of the Rat characterized with monoclonal antibodies, Chapter 6, p. 178-248 in Differentiation Antigens in Lymphohemopoietic Tissues. Eds. Miyasaka, M., Trnka, Z., *Marcel Dekker, New York and Basel.*

2. Feldmann, H., Kennard, M.L., Gabathuler, R., Yamada, T., Adams, S., and ***Jefferies, W.A.*** (1997) Serum levels of the iron binding protein p97: a novel biological marker of Alzheimer's disease. In Proceedings of the Fifth International Conference on Alzheimer's disease and related disorders by *John Wiley and Sons.*

3. ***Jefferies, W.A.***, Lizee, G., and Kennard, M.L. (1997) Creation of GPI-anchored fusion proteins. Methods in Molecular Biology, Animal Cell culture *In Humana Press Inc. U.S.A.*

4. ***Jefferies, W.A.*** (1998) Emerging Biomarkers of Alzheimer's Disease . Working Group on Biological Markers of Alzheimer's Disease, *Alzheimer's Association, Ronald and Nancy Reagan Research Institute and National Institute on Aging, NIH. U.S.A.*

4. PATENTS

Patents Issues:
1998 Novel assays to define immunological modulators
Co-discoverers: Drs. ***W. A. Jefferies***, R. Gabathuler, G.Reid, G. Kolaitis

Patent Applications Under Review:

1992 Marker for use in Diagnosing Alzheimer Disease: Potential for Therapeutic Use.
Co-discoverers: Drs. ***W. A. Jefferies***, P. McGeer, T. Yamada, and S. Rothenberger, and Mr. M. Food

1995 Creation of novel vaccines
Co-discoverers: Drs. ***W. A. Jefferies***, J. Alimonte, R. Gabathuler, G.Reid, G. Kolaitis

1995 Marker for use in Diagnosing Alzheimer Disease.
Co-discoverers: Drs. ***W. A. Jefferies*** and M., Kennard

Technology Disclosure:

1991 Peptides for use in modulating the expression of Human receptors: a general approach.
Co-discoverer: Dr. W. A. Jefferies, Mr. G. Kolaitis

Licensed Processed (1993):

Beta-emission enhancement solution
Co-discoverer: Drs. W. A. Jefferies, R. Gabathuler
Licensed to Dragon Consultant, May, 1993.

Marker for use in Diagnosing Alzheimer Disease: Potential for Therapeutic Use.
Co-discoverers: Drs. W. A. Jefferies, P. McGeer, T. Yamada, and S. Rothenberger, and Mr. M. Food
Licensed to Synapse Technologies, April, 1993

5. SPECIAL COPYRIGHTS

6. ARTISTIC WORKS, PERFORMANCES, DESIGNS

7. OTHER WORKS

8. WORK SUBMITTED (including publisher and date of submission)

Refereed papers, submitted

*1. Reid, G., Gabathuler, R., Kolaitis, G., **Jefferies, W.A.** (1998) Selective peptide translocation by a novel TAP transporter. ***Submitted to Journal of Immunology.***

2. Haidl, I., Martha, A., Blew, D., Johnson, P., and ***Jefferies, W.A., (1998)*** The cytoplasmic domain of CD45 regulates its intracellular transport and cell surface expression. ***Submitted to European J. of immunology.***

3. Zhang, Q.-J., Gabathuller, R., Saari, C.-A., Massuci, M.G., Tufaro, F., **Jefferies, W.A.**(1998) Molecular demonstration of Selective Processing of Immunodominant viral epitopes in virally infected cells. ***Submitted to Journal of Experimental Medicine.***

4. Yamada, T., Tsujioka, Y., Taguchi, T., Takahashi, M., Tsuboi, Y., Moroo, I., Yang, J., ***Jefferies, W.A.*** (1998) p97 is prodcued by plaque associated reactive microglia in Alzheimer brains and is a unique molecular hallmark of the disease. ***Submitted to Proceeding of the National Academy of Science.***

5. Hegedus, D., Pfeifer, T., Kennard, M., Gabathuler, R., ***Jefferies, W.A.***, Theilmann, D. and Grigliatti, T (1998). Intergenic Differences in the expression and the localization of human melanotrannsferrin in insect cell lines. ***Accepted, subject to minor corrections in "Protein expression and Purification".***

5. Reid, G., Gabathuler, R., Dixon, W. and ***Jefferies, W.A.*** (1998) Reduced Tapasin Expression Level in Tumour Cells is Upregulated by IFN-γ. ***Submitted to J. Immunol.***

6. Alimonti, J., Gabathuler, R., Reid, G. and ***Jefferies, W.A.*** (1998) TAP expression provides a general method for improving the recognition of malignant cell *in vivo.* ***Submitted to Nature Medicine***

7. Moise, A.R., Vitalis, T.Z., Lippe, R. and ***Jefferies, W.A.*** (1998) Resistance to TNF-α induced apoptosis and increased viral persistence are mediated by adenovirus E3/6.7K. ***Submitted to Nature.***

8. Alimonti, J., Gabathuler, R., Reid, G . and ***Jefferies, W.A.*** (1998) TAP over expression act as an adjuvant to boost anti-viral immune responses. ***Submitted to Science***

9. <u>WORK UNDERWAY</u> (including degree of completion)

2. ***Jefferies, W.A.***, Kolaitis, G., Gabathuler, R. Surrogate antigen processing as an underlying mechanism for pathogen induced autoimmunity? (1998). ***In preparation.***

3. Gabathuler, R., and ***Jefferies, W.A.*** Molecular identification of a novel gene involved in antigen processing. (1998). ***In preparation.***

4. Moroo, I., Gabathuler, Kennard, M., and Jefferies, W.A. (1998). Transcytosis of p97 across the Blood Brain Barrier. (1998). ***In preparation.***

5. Lomas, C., Gabathuler, R., Blew, D. and ***Jefferies, W.A.*** (1998) Transport elements in MHC Class I molecules control binding to the Adenovirus E3/19k protein, ***In preparation.***

6. Lippé, R. and ***Jefferies, W.A.*** (1998) New pieces to the puzzle: The adenovirus type 2 E3/6.7K protein specifically interacts with the immuno-subversive E3/19K protein. ***In preparation.***

7. Lippé, R. and ***Jefferies, W.A.*** (1998) The Adenovirus type 2 E3/19K protein does not act alone in modulating the surface expression of MHC Class I molecules. ***In preparation.***

8. Kennard, M., Alimonti, J., and ***Jefferies W.A.*** (1998). Expression of a recombinant form of phospho- lipase C for use in harvesting of GPI linked proteins ***In preparation***

9. Hsu, F., ***Jefferies, W.A.,*** (1998). Cloning and expression of murine transferrin and melanotransferrin. ***In preparation***

<u>Summary of Research Interests</u>

The work in my lab has focused on three cellular processes. We are interested in how foreign pathogens are broken down and degraded by the cellular degradation machinery and are then recognized by the host immune response. Related to this is our research on Adenovirus which possesses virulence factors which aid the virus in circumventing the host immune response. In these areas we have made contributions to identify novel cellular proteins (TAP) which degrade and transport foreign antigens into the ER where they assemble with host peptide receptors called MHC Class I molecules. Our work on Adenovirus has concentrated on characterizing two viral proteins, E3/19K and E3/6.7K, which interact to inhibit MHC Class I molecules and viral peptides from being recognized by T cells. In the future, we will define the peptide motifs which are effectively transported into the ER by the TAP molecules and hope to test the hypothesis that protease components are directly linked to the peptide transport mechanism. In addition we plan to examine the chaperone-like capabilities of the Ad proteins and the function of these molecules in an animal model of Ad infection. The third area of my research concerns a novel method by which mammalian cells acquire iron. Iron is required for cells to survive and grow. We have demonstrated that a cell surface membrane of the transferrin family of molecules called melanotransferrin or p97 is able to directly bind and transport iron into cells. We have also found that this molecule exists in two distinct forms in humans: one as a GPI-linked cell surface form and another as a soluble form. We find p97 to be rather uniquely expressed in human brain endothelium and it may act to transport iron across the Blood Brain Barrier. Furthermore, we find p97 is expressed on reactive microglia cells uniquely associated with the analyzed deposits in Alzheimer's brains. Recently, we have found soluble p97 present in elevated concentrations in AD serum. p97 may be a biochemical marker of disease progression /recovery. In the future, we plan to examine the role of p97 in BBB transcytosis. We also plan to determine if GPI-linked p97 can transport metals other than iron.

1. Most significant research contributions:

In an attempt to provide quantitation of the ranking of journals I have recently published, I received copies of the rank order of journals from each subject area. This rank order was published in the *SCI Journal Citation Reports.* Omitting the rankings of journals which only publish review articles, I have stated the rank order of each paper I have published. Please see attached of the photocopy of the SCI rankings.

Underlined denotes those supervised by W.A.J.

Summary of manuscript deemed most important:

1. ***Jefferies, W.A.***, and Burgert, H.-G. (1990) E3/19k from Adenovirus 2 is an immunosubversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. J.Exp. Med. 172, 1653-1664.

Area: Immunology
Rank Order: 1 of 73 Journals (See appendix 1)

-This paper described that the structures within MHC Class I molecules which control intracellular transport also contribute to the binding of the Adenovirus E3/19k protein which subverts the immune response by inhibiting the expression of the MHC molecules which would otherwise present adenoviral peptides to T cells and allow the destruction of virally infected cells. . It has wide implications for both viral persistence and the assembly of MHC molecules.

2. Lippé, R., Luke, E., Kaun, Y.-T., Lomas, C., and ***Jefferies, W.A.*** (1991) Adenovirus-2 inhibits the phosphorylation of human major histocompatibilty complex antigens. J. Exp. Med. 174: 1159-1166.

Area: Immunology
Rank Order: 1 of 73 Journals

This paper describes the first persistent viral infection in which the phosphorylation of MHC Class I molecules is inhibited and demonstrates that the E3/19k proteins localizes MHC Class I molecules in the ER.

3. Matsuse, T., Hayashi, S., Kuwano, K., Keunecke, H., ***Jefferies, W.A.*** and Hogg, J.C. (1992).The Adenoviral early glycoprotein E1a but not the E3/19k gene is amplified in frozen lung tissue from patients with Chronic Obstructive Pulmonary Syndrome. Am. Rev. Respir Dis. 146: 177-184

Area: Respiratory System
Rank Order: 1 of 20 Journals

This paper provides evidence for the link between Chronic Obstructive Pulmonary Syndrome and adenovirus infection.

4. Lippé, R., Kolaitis, G., Michaelis, C., Tufaro, F., and ***Jefferies, W.A.*** (1991) Distinguishing between the requirements for viral versus allogeneic cytotoxic T cell recognition. J. Immunol. 150, 3170-3179..

Area: Immunology
Rank Order: 2 of 73 Journals

This paper provides the first evidence that viral antigens have distinct methods for entering the exogenous antigen processing pathway.

5a. ***Jefferies, W.A.***, Kolaitis, G., Gabathuler, R. (1993) The Interferon-gamma induced recognition of the antigen processing variant CMT.64 by cytolytic T cells can be replaced by sequential addition of B2-microglobulin and antigenic peptides. J. Immunol. 151: 2974-2985.

Area: Immunology
Rank Order: 2 of 73 Journals

5b. Gabathuler, R., Reid, Gregor, Kolaitis, G., Driscoll, J., and ***Jefferies, W.A.*** (1994) Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. J. Exp. Med. 180: 1415-1425.

Area: Immunology
Rank Order: 1 of 73 Journals (See appendix 1)

5c. Marusina, K., Reid, G., Gabathuler, R., ***Jefferies, W.A.***, and Monaco, J.J. (1997) Novel peptide-binding proteins and peptide transport in normal and TAP-deficient microsomes. Biochemistry 36: 856-863.

Area: Biochemistry and Molecular Biology
Rank Order: 9 of 151 Journals

5d. Reid, G., Gabathuler, R., Kolaitis, G., ***Jefferies., W.A.*** (1994) Selective peptide translocation by a novel TAP transporter. ***In revision.***

-These four manuscripts demonstrate that TAP -1 alone is able to deliver certain viral proteins into the ER where they nucleate the assembly of MHC Class I molecules. These experiments change the paradigm for peptide transport into the ER. Furthermore, they also demonstrate that a potential method to allow cancer cells to evade removal by the host immune response is to down regulate MHC expression via dampening the expression of the TAP molecules. **Marusina et al., 1996**, describes novel proteins which may be involved in TAP dependent and TAP independent transport into the ER.

6. Food, M., Rothenberger, S., Gabathuler, R., Haidl, I., Reid, G., and ***Jefferies, W.A.*** (1994) Transport and expression in human melanomas of a transferrin-like glycosylphosphatidylinositol anchored protein. J. Biol. Chem. 269: 3034-3040.

Area: Biochemistry and Molecular Biology
Rank Order: 5 of 151 Journals

-This paper describes for the first time that the p97 molecule is linked to the cell surface via a glycolipid anchor and exists as a secreted soluble protein as well.

7. Kennard, M., Richardson, D. Gabathuler, R., Ponka, P., ***Jefferies, W.A.*** (1995) A novel iron uptake mechanism mediated by GPI-anchored human p97. EMBO Journal, 4178-4186.

Area: Biochemistry and Molecular Biology
Rank Order: 2 of 151 Journals

-This paper identifies in mammals only the second route by which cells acquire iron. The fact that iron uptake is facilitated by a glycolipid anchored molecule implies that this is a novel method of internalization. I think this paper will stand as an important contribution in the area of metal transport.

8. Rothenberger, S., Food, M.R., Gabathuler, R., Kennard, M., Yamada, T., Yasuhara, O., McGeer, P.L., ***Jefferies, W.A.*** Coincident expression and distribution of melanotransferrin and transferrin receptor in human brain capillary endothelium. Brain Research, 712. 117-121

-This paper describes the localization of the p97 molecule in the brain endothelium, in the same location as the transferrin receptor **(Jefferies et al., 1984)** and suggest this molecule may be directly involved in the deliver of iron into or out of the brain. This contention is supported by our recent unpublished data.

9. ***Jefferies, W.A.***, Food, M.R., Gabathuler, R., Kennard, M., Rothenberger, S., Yamada, T., Yasuhara, O., McGeer, P.L. (1995) Reactive microglia specifically associated with amyloid plaques in Alzheimer's disease brain tissue express melanotransferrin. Brain Research, 712, 122-126.

-This manuscript identifies p97 as a unique marker of those microglia associated with the beta-amyloid deposits in Alzheimer's brains. It was not found expressed in the brains of patients with other disease of the brain and it was not found in microglia not associated with plaques. This is the first marker of its kind and we have used this finding to develop an AD serum monitoring assay which appears to be able to identify AD patients from controls. In

addition, these findings imply that p97 has a role in metal uptake in these microglia cells and we plan to pursue this hypothesis.

10. ***Jefferies, W.A.***, Gabathuler, R., Rothenberger, S., Food, M., Kennard, M. Pumping iron in the 90's. Pumping iron in the 90's. (1996). Trends in Cell Biology 6, 223-228.

-This is a refereed review commissioned to undertake by the Editors of *Trends in Cell Biology*. I believe that a review of the iron transport field with particular emphasis on the p97 molecule and our work, will help to further popularize this are of research. This journal is widely read.

11. Kennard, M., Feldman, H., Yamada, T., and ***Jefferies, W.A.*** (1996) Serum levels of the iron binding protein p97 are elevated in Alzheimer's disease. Nature Medicine 11:1230-1235.

-This paper describes the first stand alone protein marker, p97, which appears to be elevated in individuals with Alzheimer's Disease. This marker appears to increase in the serum of patients and mirrors disease progression.

12. Gabathuler, R., Alimonte, J., Qian, Q.-J., Kolaitis, G., Reid, G., ***Jefferies, W.A.*** (1998) Surrogate antigen processing and the alternative fate of antigenic peptides within the secretory pathway Journal of Cell Biology 140, 17-27.

-This manuscript describes a novel form of antigen processing which results from the secretion of antigenic peptides from virally infected cells and the subsequent binding to uninfected cells. This results in the killing of the uninfected cells by virus specific CTL. These phenomena could explain some aspects of auto-immunity and self-tolerance.

THIS IS **EXHIBIT "B"** MENTIONED AND REFERRED TO IN THE DECLARATION OF **WILFRED A. JEFFERIES**

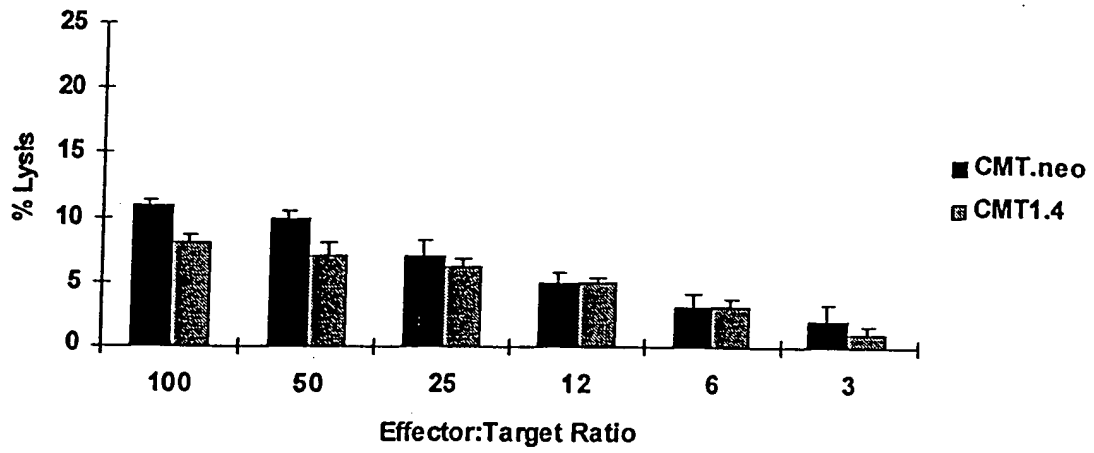
Wilfred A. Jefferies

4/3/99

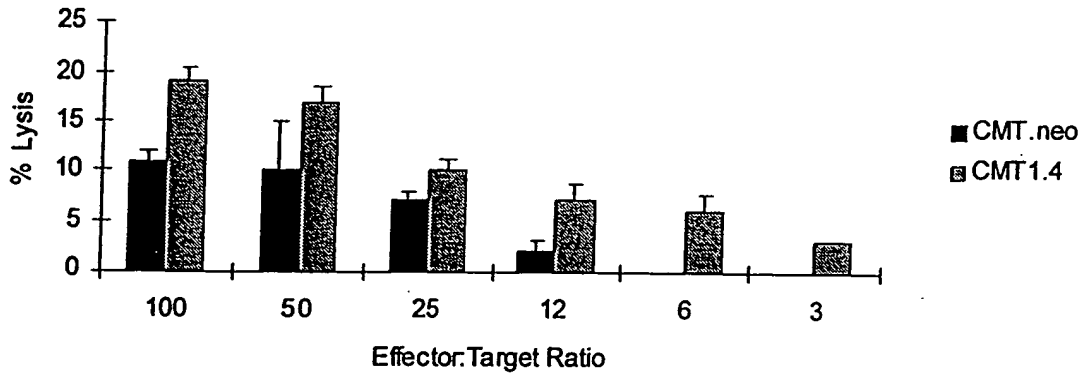
Date

FIGURE 1

A



B



Specificity of splenocytes from mice injected with CMT.neo or CMT1.4

The specificity of splenocytes from a mouse injected with A) CMT.neo or B) CMT1.4 was determined in a CTL assay against the ^{51}Cr labeled targets CMT.neo, and CMT1.4. Upon removal the splenocytes were cultured with stimulator cells at a 3:1 ratio. The stimulator cells were prepared by incubating CMT1.4 or CMT.neo cells with 30 μg/ml mitomycin C under hypoxic conditions. After a 2 hour incubation the cells were γ-irradiated (10,000 Rads) and washed three times before addition to the splenocyte culture. CMT.neo splenocytes received CMT.neo stimulators, whereas CMT1.4 splenocytes received CMT1.4 stimulators. 6 days after *in vitro* stimulation the splenocytes were tested in a standard 4 hour ^{51}Cr release assay.

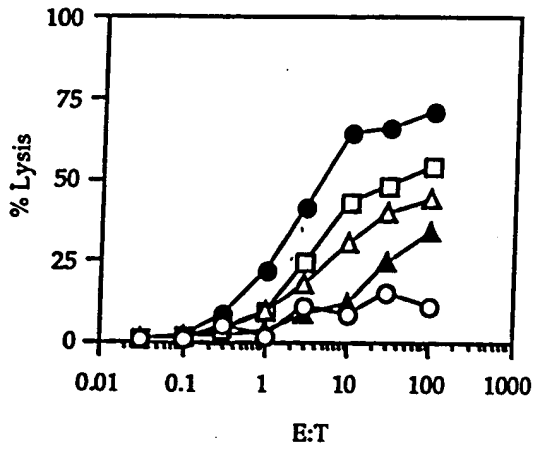
THIS IS **EXHIBIT "C"** MENTIONED AND REFERRED TO IN THE DECLARATION OF **WILFRED A. JEFFERIES**

Wilfred A. Jefferies

4/3/99

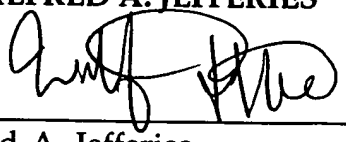
Date

FIGURE 2



Allogeneic H-2^d anti H-2^b CTL can lyse single and double rTAP-1/2 transfected CMT.64 cell lines. BALB/c mice were immunized *in vivo* with B6 splenocytes. Responder spleen cells were then stimulated *in vitro* with irradiated B6 splenocytes and used in a CTL assay. CMT.IFN are CMT.64 cells treated for 24h with mouse gamma interferon (200 U/ml) and further used in a CTL assay. CMT.64 (—o—); CMT 1-4 (—Δ—); CMT 2-10 (—▲—); CMT 12-12 (—□—); CMT.IFN (—●—) .

THIS IS EXHIBIT "D" MENTIONED AND REFERRED TO IN THE DECLARATION OF **WILFRED A. JEFFERIES**



Wilfred A. Jefferies

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Date

TABLE 1

Table 1 : Lysis of the rTAP1/2 transfected CMT cells by the LD cultures obtained from BALB/c mice immunized with the various transfectants

Targets cells	LDA 12-12	LDA 1-4	LDA 2-10
CMT 64	0[a]	0	0
CMT 1-4	21	51	46
CMT 2-10	20	0	6
CMT 12-12	24	0	0

a) represent the number of positive wells obtained. 96 wells were tested. Well was considered positive when the cpm was 5x the SD above the spontanous release

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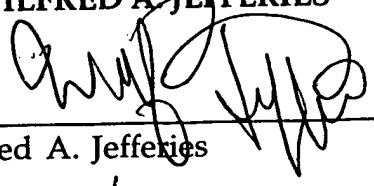

Wilfred A. Jefferies

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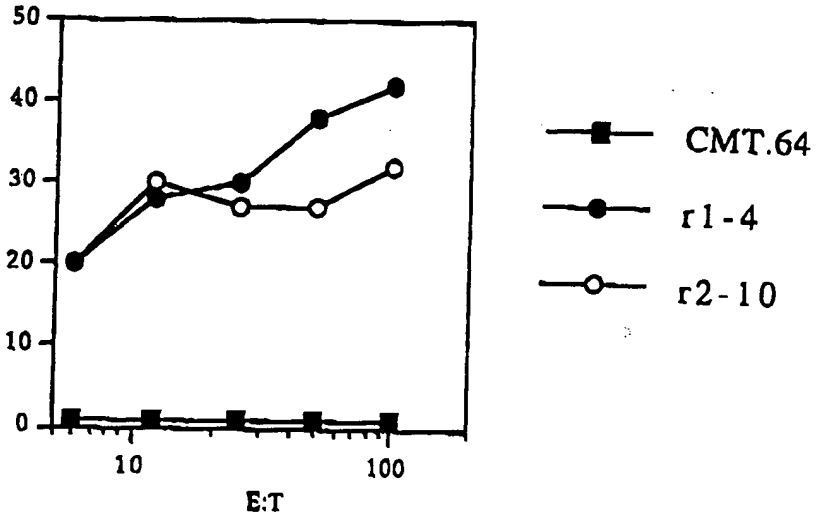
Date

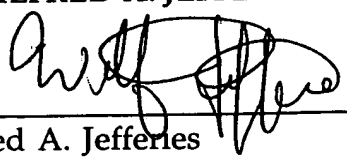
FIGURE 3



Virus presentation by CMT r1 and r2 clones.

The ability of CMTr1 and CMTr2 clones to present Infl.A was compared to the parental CMT.64 cell line. The results shown using clones r1-4 and r2-10 are representative of each class of transfectant. The target cells are incubated with Infl.A for 4 hours (200HA units/10^6 cells) prior to a 4 hour release assay in the presence of virus specific CTL.

THIS IS **EXHIBIT "F"** MENTIONED AND REFERRED TO IN THE DECLARATION OF **WILFRED A. JEFFERIES**



Wilfred A. Jefferies

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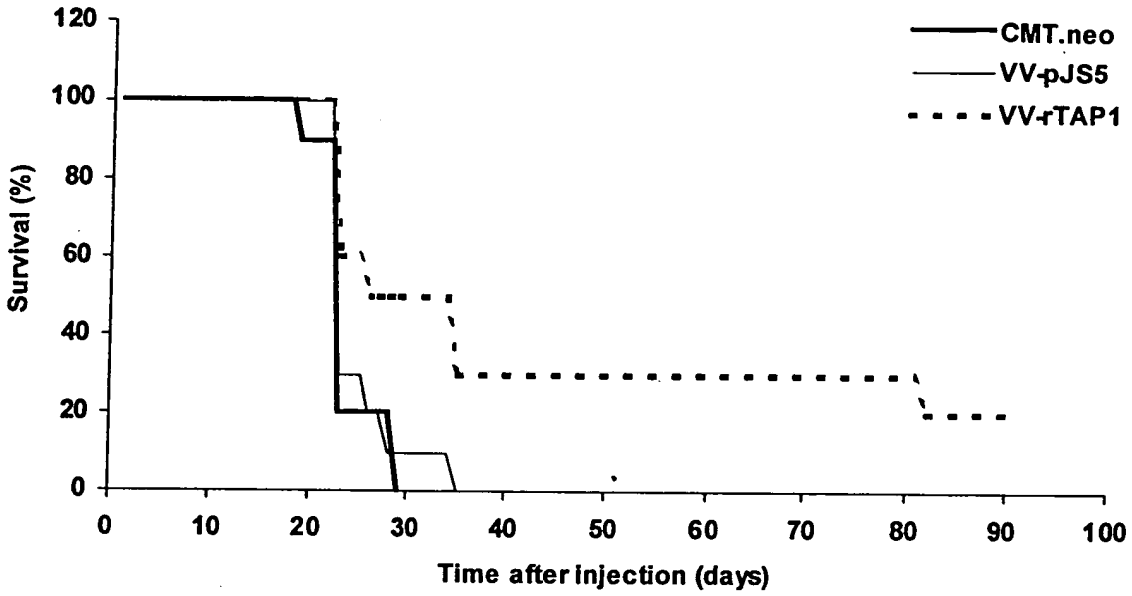
Date

FIGURE 4



VV-rTAP1 therapy of CMT.neo burdened mice

Three groups of 10 C57B1/6 mice were injected ip. with $5X10^5$ CMT.neo in PBS. Two of the three groups received either VV-pJS5, or VV-rTAP1 treatment. In the two rVV treated groups, the CMT.neo cells were injected into the mice then treated ip. at 24 hours, and at 2 weeks with 10^6 pfu rVV in PBS containing 2% C57B1/6 mouse serum.

The EMBO Journal vol.15 no.13 pp.3247–3255, 1996

Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47

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K.Ahn and T.H.Meyer contributed equally to this work

The immediate early protein ICP47 of herpes simplex virus (HSV) inhibits the transporter for antigen processing (TAP)-mediated translocation of antigen-derived peptides across the endoplasmic reticulum (ER) membrane. This interference prevents assembly of peptides with class I MHC molecules in the ER and ultimately recognition of HSV-infected cells by cytotoxic T-lymphocytes, potentially leading to immune evasion of the virus. Here, we demonstrate that recombinant, purified ICP47 containing a hexahistidine tag inhibits peptide import into microsomes of insect cells expressing human TAP, whereas inhibition of peptide transport by murine TAP was much less effective. This finding indicates an intrinsic species-specificity of ICP47 and suggests that no additional proteins interacting specifically with either ICP47 or TAP are required for inhibition of peptide transport. Since neither purified nor induced ICP47 inhibited photocrosslinking of 8-azido-ATP to TAP1 and TAP2 it seems that ICP47 does not prevent ATP from binding to TAP. By contrast, peptide binding was completely blocked by ICP47 as shown both by photoaffinity crosslinking of peptides to TAP and peptide binding to microsomes from TAP-transfected insect cells. Competition experiments indicated that ICP47 binds to human TAP with a higher affinity (50 nM) than peptides whereas the affinity to murine TAP was 100-fold lower. Our data suggest that ICP47 prevents peptides from being translocated by blocking their binding to the substrate-binding site of TAP.

Keywords: ABC-transporter/antigen presentation/antigen processing/herpes simplex/immune evasion

Introduction

Viruses have evolved means to evade immune defense, in particular, recognition by $CD8^+$ cytotoxic T-lymphocytes (Koup, 1994). Such T-cells identify infected cells via T-cell receptors specific for class I major histocompatibility complex (MHC) antigens presenting antigen-derived peptides (Townsend and Bodmer, 1989). Several virus species belonging to the adenovirus as well as herpes virus families specifically intervene with the class I MHC antigen processing and presentation pathway (reviewed by Hill and Ploegh, 1995). In this pathway three components, class I heavy chain, β2-microglobulin and peptides, have to be generated, assembled and transported to the cell surface (Jackson and Peterson, 1993). Both heavy chain and β2-microglobulin are translocated into the endoplasmic reticulum (ER) during synthesis. By contrast, antigens are thought to be degraded to peptides in the cytosol by an evolutionary conserved proteolytic machinery which involves conjugation of ubiquitin to target proteins and subsequent proteolysis by the multisubunit proteasome particle (Michaelek *et al.*, 1993; Rock *et al.*, 1994). Therefore, assembly of the trimeric class I MHC complex requires import of cytosolically produced peptides into the ER lumen. For this purpose, specific transport molecules that reside in the ER membrane have evolved (reviewed by Howard, 1995). Peptide transporters consist of two polypeptide chains, TAP1 and TAP2, both of which are encoded in the class II region of the MHC (Deverson *et al.*, 1990; Monaco *et al.*, 1990; Spies *et al.*, 1990; Trowsdale *et al.*, 1990). TAP1 and TAP2 belong to a class of ATP-driven pumps, the ATP-binding-cassette family of transmembrane transporters, which includes bacterial transporters as well as the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (Higgins, 1992). Analysis of the peptide spectrum translocated by TAP indicated that TAP preselects peptides within a size range similar to the 8–10mer peptides bound to class I MHC molecules (Momburg *et al.*, 1994b; Schumacher *et al.*, 1994; Van Endert *et al.*, 1994). Similarly, TAP from different species (human and mouse) or haplotypes within a species (rat), display a limited specificity for peptide sequences corresponding to the peptides bound by the respective class I MHC molecules (Powis *et al.*, 1992; Heemels *et al.*, 1993; Momburg *et al.*, 1994b). Furthermore, a physical association of empty class I MHC heterodimers with TAP has been demonstrated (Ortmann *et al.*, 1994; Suh *et al.*, 1994). Upon translocation of peptides fitting the peptide-binding motif of the TAP-bound class I MHC molecule, the trimeric complex is released from TAP and transported to the cell surface.

Although both adenovirus and herpes virus prevent class I MHC molecules from appearing at the cell surface, their strategies differ. Adenovirus types 2 and 5 retain fully assembled trimeric class I molecules in the ER by expressing the class I binding, ER-resident protein E19 (Andersson *et al.*, 1985; Burgert and Kvist, 1985). By contrast, in HSV-1-infected cells, class I MHC molecules are not loaded with peptides and remain in the ER as a

consequence of such incomplete assembly (Hill *et al.*, 1994; York *et al.*, 1994). Thereby, expression of the immediate early gene ICP47 suffices to prevent class I MHC assembly (York *et al.*, 1994). Recently, we and others showed that ICP47 inhibits peptide translocation across the ER membrane (Früh *et al.*, 1995; Hill *et al.*, 1995). Furthermore, a direct interaction between TAP and ICP47 was observed. These findings suggested that ICP47 prevents peptide loading of class I MHC molecules by inhibiting TAP-mediated peptide translocation across the ER membrane.

It remains to be determined by which molecular mechanism ICP47 achieves such inhibition of peptide translocation. The molecular events of translocation, as well as the structural features of TAP, are only poorly understood. However, by reconstituting peptide translocation in insect cells (Meyer *et al.*, 1994) it was possible to distinguish experimentally between several sequential translocation steps (Van Endert *et al.*, 1994). A necessary requirement for peptide translocation is hydrolysis of ATP (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993; Shepherd *et al.*, 1993). By contrast, binding of peptides to TAP is ATP-independent (Androlewicz and Cresswell, 1994; Van Endert *et al.*, 1994; Uebel *et al.*, 1995) and, vice versa, ATP binding occurs in the absence of peptides (Müller *et al.*, 1994; Wang *et al.*, 1994) indicating that these are separate steps preceding peptide translocation. We have therefore examined whether peptide translocation is inhibited as a consequence of ICP47 inhibiting ATP binding or peptide binding. Furthermore, by using purified ICP47 and TAP-expressing insect cells we were able to address the question whether additional molecules are necessary for TAP inhibition by ICP47. Finally, we have compared the specificity of ICP47 binding as well as peptide binding to human versus murine TAP. We discuss a potential mechanism for TAP inhibition by ICP47.

Results

Purified recombinant ICP47 inhibits peptide transport

To facilitate the purification of recombinant ICP47 we fused a hexahistidine tag to the amino-terminus of ICP47 which allows purification over NTA–agarose (Hochuli *et al.*, 1988). His-ICP47 was highly overexpressed in *Escherichia coli* after 2 h induction with IPTG (Figure 1A) and was purified by affinity chromatography using NTA–agarose in urea-containing buffer, followed by an additional reverse-phase HPLC step. The resulting product appeared as a single protein band in Coomassie-stained SDS–PAGE gels (Figure 1A) and as a single peak in HPLC analysis (Figure 1B). His-ICP47 was renatured by dialysis against water.

Next, we tested whether such purified His-ICP47 would inhibit peptide transport. Since ICP47 expressed in HeLa cells prevented TAP-dependent import of labeled peptides into the ER (Früh *et al.*, 1995), we added different concentrations of His-ICP47 to streptolysin O (SLO)-permeabilized HeLa cells and assayed peptide translocation (Figure 2A). We used an assay originally described by Neefjes *et al.* (1993) whereby ER-specific glycosylation of peptides containing a glycosylation signal can be monitored by recovering such peptides with the lectin

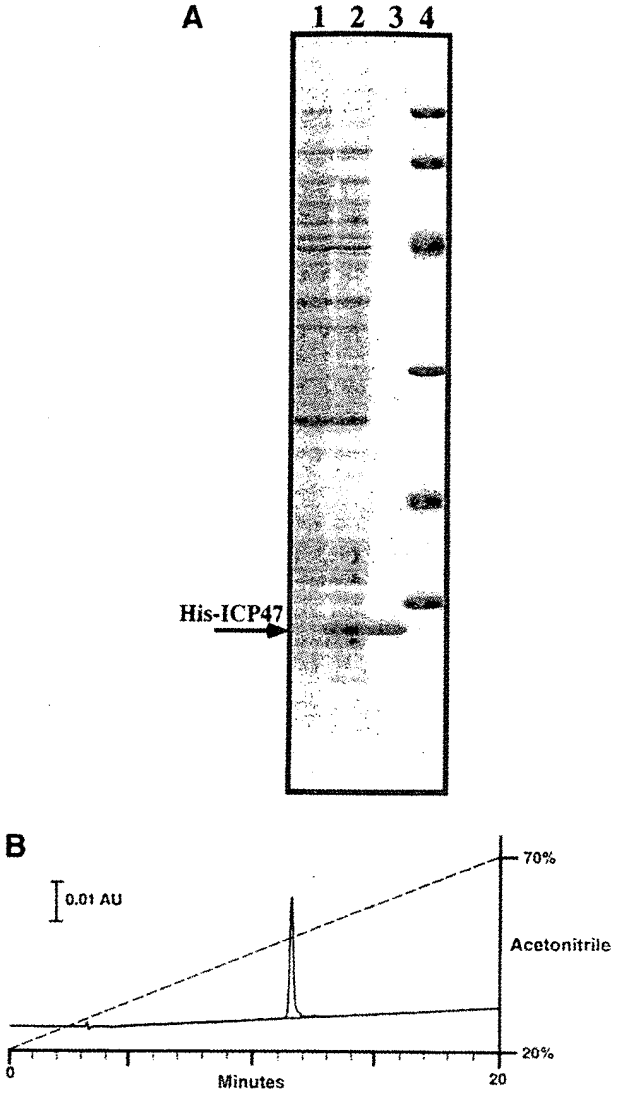

Fig. 1. Purification of His-ICP47. (A) SDS–PAGE analysis of total cell lysates of *E.coli* strain Blys transformed with His-ICP47.pRK171 either without (lane 1) or after 2 h induction with 1 mM IPTG (lane 2). Lane 3 contains 5 μg of His-ICP47 purified as described in Materials and methods. Lane 4: molecular weights (in kDa) of standard proteins (Bio-Rad) are (from top): 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4. The predicted molecular weight of His-ICP47 is 11.179 kDa. (B) Reverse-phase HPLC profile of purified His-ICP47. The purified material was eluted from a C18 column by using a 20–70% acetonitrile gradient. AU, absorption unit at 214 nm.

concanavalin A (ConA). In the presence of micromolar concentrations of His-ICP47, recovery of glycosylated peptides was reduced to levels similar to those observed in the absence of ATP (Figure 2A). These results suggested that purified renatured His-ICP47 was capable of inhibiting peptide translocation completely. Furthermore, no loss in activity was observed for His-ICP47 heat-treated at 100°C for 5 min (not shown). Since it had been observed previously that longer peptides can be degraded to shorter derivatives during the course of the peptide transport assay (Momburg *et al.*, 1994a), it was possible that not His-ICP47 (99 amino acids), but a peptidic breakdown product was responsible for the observed inhibition of peptide translocation. Therefore, we analyzed the stability of His-ICP47 in the presence of SLO-permeabilized HeLa cells

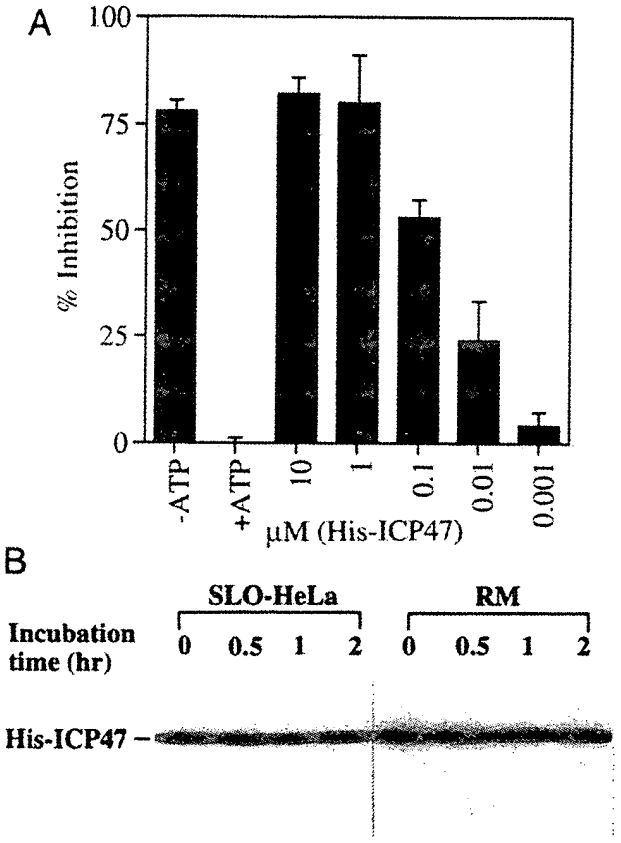

Fig. 2. His-ICP47 inhibits peptide translocation into permeabilized HeLa cells. (**A**) Peptide translocation into the ER of 2×10^6 streptolysin O-permeabilized HeLa cells was assayed using peptide RYWANATRST (R-10-T). (Error bars indicate standard deviation of duplicate measurements.) For inhibition experiments cells were incubated with the indicated concentrations of His-ICP47 for 10 min at 37°C before peptide addition. Recovery of glycosylated peptides by ConA–Sepharose is expressed as percent of peptides recovered in the absence of ICP47 (+ATP). Without inhibitor, 0.42 ± 0.05% of peptides (8408.5 c.p.m.) were recovered. As control, ATP was omitted and cells were treated with apyrase (–ATP). (**B**) His-ICP47 is not degraded under assay conditions. His-ICP47 was iodinated by the iodogen method (Judd, 1990). 0.6 µg labeled material was incubated at 37°C for the indicated time periods with either 2×10^6 streptolysin O-permeabilized HeLa cells (SLO-HeLa) or 15 µl crude microsome preparations (RM) of Sf9 insect cells expressing hTAP, in 100 or 150 µl assay buffer, respectively. The reaction mixture was separated on a 10–20% SDS–PAGE gel. Exposure time of the autoradiograph was 48 h.

or crude microsomal membrane fractions isolated from insect cells used in experiments described below. As shown in Figure 2B, the amount of full-length His-ICP47 remained unchanged during 30 min to 2 h at 37°C. We conclude that during the 20-min incubation period of the peptide transport assay full-length His-ICP47 was responsible for the observed inhibition.

Using radiolabeled His-ICP47 we also tested whether ICP47 itself could be translocated into the ER. Since ICP47 contains the potential glycosylation site NAS at position 64 (Watson and Vande Woude, 1982), it is possible to assay translocation of ICP47 analogous to peptides. However, we did not recover above-background levels of His-ICP47 by ConA precipitation and did not observe any difference of radioactivity associated with microsomes in the presence and absence of ATP, which suggests that ICP47 is not transported by TAP (not shown).

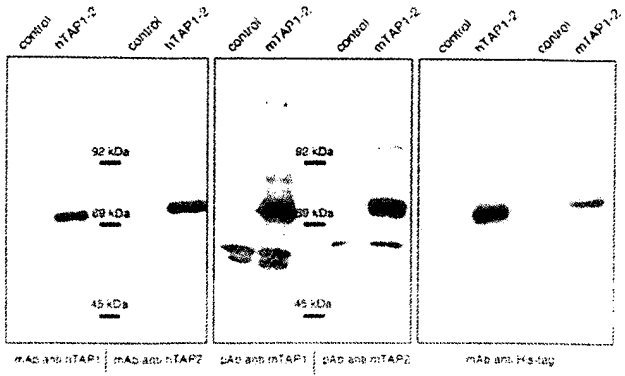

Fig. 3. Expression of human and murine TAP in insect cells. 5 µg of total microsomal protein from BV-hTAP-infected (hTAP1-His-2) or mTAP-transfected (mTAP1-His-2) PS5 cells were loaded on SDS–PAGE (7.5%) and analyzed by immunoblot using monoclonal antibodies against hTAP1 (mAb 148.3) and hTAP2 (mAb 429.4), polyclonal antisera against mTAP1 and mTAP2, or a monoclonal antibody against the hexahistidine tag. Control samples containing either wild-type baculovirus-infected Sf9 cells or non-induced PS5 cells were loaded next to the respective TAP-containing samples. The predicted molecular weights of the TAP polypeptides range from 71 to 77 kDa. Additional proteins recognized by the polyclonal antisera against mTAP are non-specific, since they are also present in the control lanes.

ICP47 is sufficient to inhibit peptide transport by human but not murine TAP

To test whether any specific proteins besides TAP would be required for ICP47 function we examined whether isolated His-ICP47 would be able to block peptide translocation into microsomes isolated from TAP-expressing insect cells. By expressing both human and murine TAP we further addressed the question whether the previously observed inability of ICP47 to block murine TAP function (York *et al.*, 1994; Früh *et al.*, 1995) was due to a preferred interaction of ICP47 with human TAP molecules. Human TAP was expressed in Sf9 cells using the baculovirus expression system (Meyer *et al.*, 1994). Murine TAP was expressed in *Drosophila melanogaster* SC2 cells under control of the metallothionine promoter in the stable cell line PS5. Microsomes from both infected Sf9 cells and induced PS5 cells were isolated and tested for expression of TAP1-His and TAP2 using specific antisera. Non-infected Sf9 or non-induced PS5 cells were used as control. Upon baculovirus infection or induction with $CuSO_4$, expression of human and respectively murine TAP1-His and TAP2 could be detected with TAP-specific antibodies in an immunoblot (Figure 3). In addition, since TAP1-His of both species was fused to a hexahistidine tag (see Materials and methods), TAP1-His was identified by a hexahistidine-specific antibody (Figure 3). Thereby, a stronger signal was obtained from baculovirus-expressed human TAP1-His, suggesting higher expression levels of hTAP. However, microsomes (50 µg protein) of both murine and human TAP-containing insect cells translocated ~1.5% of input peptide R-10-T (Meyer *et al.*, 1994) under standard conditions (Neefjes *et al.*, 1993) whereas non-induced PS5 or non-infected Sf9 cells translocated less than 0.1% peptide.

We compared the inhibitory effect of His-ICP47 on peptide translocation into human and murine TAP-containing insect cell microsomes (Table I). After preincubat-

Table I. Relative efficiencies of peptides and His-ICP47 in competition of peptide transport

Competitor	Reporter	Sequence	IC_{50}[a]	
			Human TAP	Mouse TAP
R-10-T	R-10-T	RYWANATRST	1.13 ± 0.13	0.78 ± 0.17
His-ICP47	R-10-T	RYWANATRST	1.99 ± 0.21	55.6 ± 3.4
R-8-L	R-8-L	RYNATRGL	1.15 ± 0.02	0.65 ± 0.05
His-ICP47	R-8-L	RYNATRGL	0.96 ± 0.13	61.8 ± 3.5

[a]Molar excess required for 50% inhibition of reporter peptide (250 nM).

ing microsomes for 5 min with the indicated molar excess of His-ICP47 or the respective competitor peptides, translocation of reporter peptides was monitored. Half-maximal inhibition (IC_{50}) of peptide translocation by human TAP was achieved at a molar excess of 1 to 2 of His-ICP47, depending on the reporter peptide (Table I). Although both reporter peptides used, R-10-T and R-8-L, were transported equally well by human and murine TAP the IC_{50} values obtained with His-ICP47 differed greatly. Human TAP was inhibited by ICP47 within the same concentration range observed for unlabeled peptides (Figure 4). By contrast, 60-fold molar excess of His-ICP47 over reporter peptide was required to inhibit 50% of peptide import into insect cell microsomes containing murine TAP, whereas IC_{50} values for unlabeled peptides were similar to human TAP (Table I). However, as shown in Figure 4, the IC_{50} values for murine TAP were derived from experiments in which complete inhibition of peptide transport was not observed. Since His-ICP47 precipitated out of solution above 10 μM, we did not analyze peptide transport at higher His-ICP47 concentrations. However, by lowering the reporter peptide concentration we measured inhibition of murine TAP at 500-fold molar excess of His-ICP47. We observed that even at such a high molar ratio, His-ICP47 was not able to inhibit peptide translocation by murine TAP completely (data not shown). Thus, we conclude that ICP47 is unable to completely inhibit peptide transport by murine TAP transporters. Moreover, our results show that ICP47 by itself is sufficient to efficiently inhibit peptide transport by human TAP, since it is unlikely that microsomes from insect cells provide any specific protein required for the function of either TAP or ICP47. Although specific proteins binding to ICP47 or to TAP might exist, they do not seem to be essential for this interaction.

ICP47 does not inhibit ATP binding to TAP

Since ATP binding and hydrolysis are essential for TAP function (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993; Shepherd *et al.*, 1993), it seemed possible that ICP47 interferes with ATP binding. The nucleotide-binding domains of both TAP1 and TAP2 can be photoaffinity labeled with 8-azido-[α-^{32}P]ATP (Müller *et al.*, 1994; Wang *et al.*, 1994; Russ *et al.*, 1995). Using the same ATP analog we tested whether ICP47 interferes with ATP binding to TAP. We isolated microsomes from ICP-O20 cells, a stable HeLa cell line in which ICP47 expression is inducible by removal of tetracycline (Früh *et al.*, 1995). After 10 min incubation on ice followed by UV-irradiation, microsomes were lysed and both TAP1 and TAP2 were immunoprecipitated using specific antibodies. Consistent

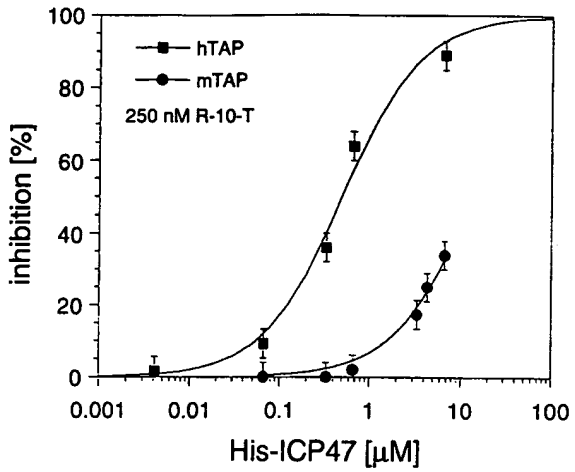

Fig. 4. His-ICP47 inhibits peptide translocation by human TAP at lower concentrations compared with murine TAP. Peptide transport into insect cell microsomes containing either human TAP (■) or mouse TAP (●) was assayed as described in Materials and methods using reporter peptide R-10-T at a concentration of 250 nM. The concentration of His-ICP47 is plotted against percent inhibition of transport measured in the absence of inhibitor. His-ICP47 was added simultaneously with reporter peptides in these experiments which were performed in duplicates (± SD). In the absence of inhibitor, 1.5 ± 0.05% of input peptide (1.3×10^6 c.p.m.) was recovered after ConA–Sepharose precipitation.

with our previous findings both TAP1 and TAP2 were crosslinked by azido-ATP, indicating that both subunits bound azido-ATP, and crosslinking to both subunits was inhibited by excess of ATP (Figure 5A). However, no difference in azido-ATP labeling was observed between microsomes isolated from ICP47-containing cells (–tet) or microsomes from ICP-O20 cells cultured in the presence of tetracycline. The possibility that ICP47 dissociated from the ATP-binding domain during microsome preparations is unlikely, because even in the presence of detergents ICP47 remains bound to TAP (Früh *et al.*, 1995). Furthermore, addition of 10 μM His-ICP47 to microsomes isolated from HeLa cells did not affect ATP binding to either TAP1 or TAP2 (Figure 5B), although complete inhibition of peptide transport was observed at this concentration of His-ICP47, as described above. We interpret these findings as evidence against ICP47 interfering with ATP binding.

ICP47 inhibits peptide binding to TAP

In addition to the ATP-binding sites which are present in two copies per heterodimer, TAP molecules seem to possess a single peptide-binding site shared between the two subunits (Androlewicz *et al.*, 1994). To study whether ICP47 interferes with peptides binding to this site we

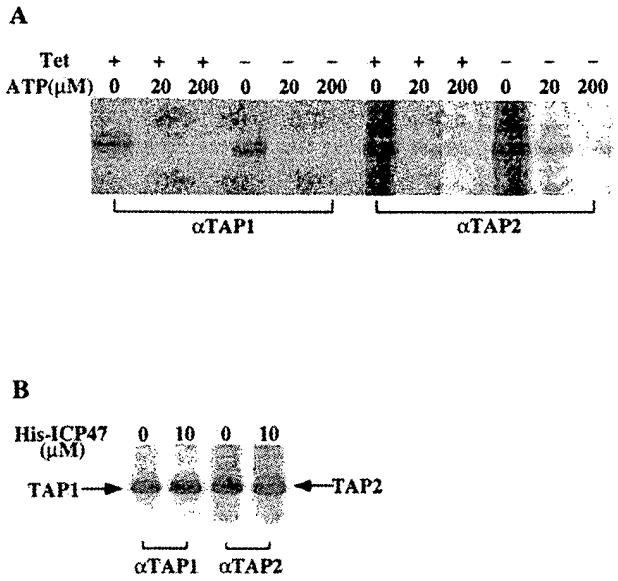

Fig. 5. Photoaffinity labeling of TAP with 8-azido-ATP is not affected by ICP47. Autoradiographs of immunoprecipitations of TAP1 or TAP2 from microsomes photocrosslinked to [α-^{32}P]8-azido-ATP. (A) Expression of ICP47 in stably transfected ICP-O20 cells (Früh *et al.*, 1995) was either induced by removal of tetracycline (–) or not induced by continuous culture with 1 μg/ml tetracycline (+) before preparation of microsomes. (**B**) HeLa cell microsomes were incubated for 10 min with or without 10 μM of purified His-ICP47 before photocrosslinking. hTAP1 was immunoprecipitated by mAb 148.3 (Meyer *et al.*, 1994); hTAP2 was immunoprecipitated with mAb 429.4 (Van Endert *et al.*, 1994) followed by SDS–PAGE using a 10–15% gradient gel. Autoradiographs were exposed for 7 days in (A) and 4 days in (B). In (A), binding of azido-ATP to TAP1 and TAP2 was inhibited using either 20 or 200 μM ATP as indicated.

examined the binding of a photoreactive analog of the peptide KYWANATRSGL to microsomes of ICP-O20 cells. This peptide carries the crosslinker HSAB at the α-amino group of the amino-terminal lysine. 2.5 μM labeled peptides were incubated with microsomes and, after UV-crosslinking, both TAP1 and TAP2 were immunoprecipitated using respective antibodies and separated by SDS–gel electrophoresis (Figure 6A). Both subunits were found to be crosslinked to the labeled peptide although TAP2 seemed to be labeled more intensely. Preferential crosslinking to either TAP1 or TAP2 seems to depend on the individual peptide and the relative position of the crosslinker (Androlewicz *et al.*, 1994). When ICP47 had been induced by tetracycline removal before preparing microsomes, a strong decrease of photoaffinity labeling was observed for both TAP1 and TAP2 (Figure 6A). Thus, ICP47 seems to remain bound to microsomes during preparation, thereby preventing peptide binding to TAP. In another series of experiments, we added purified His-ICP47 to microsomes isolated from HeLa cells before UV-crosslinking. Subsequent immunoprecipitation of TAP1 demonstrated that His-ICP47 inhibited peptide binding (Figure 6B). Scanning of the TAP1 labeling intensity indicates half-maximal inhibition of peptide binding at a concentration of His-ICP47 of ~250 nM (Figure 6C). In summary, both transfected ICP47 and added purified ICP47 prevented photocrosslinking of peptides to TAP.

To quantify the competition between peptides and ICP47 for binding to TAP, we used a recently developed assay

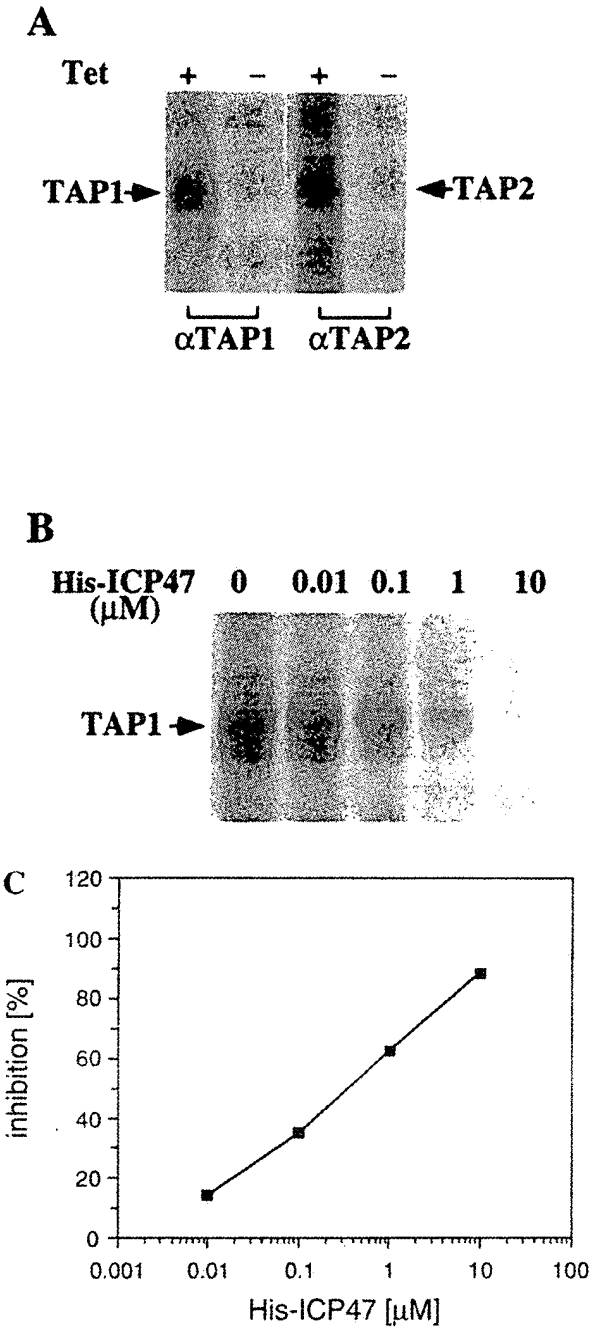

Fig. 6. ICP47 inhibits TAP binding of photoreactive peptides. (**A**) Iodinated peptide KYWANATRSGL (2.5 μM) containing the photoreactive crosslinker HSAB at its amino-terminus was added to microsomes which were isolated from either induced or non-induced ICP-O20 cells (– or + tetracycline). (**B**) His-ICP47 was added to microsomes isolated from HeLa cells at the indicated concentrations 10 min before adding peptides. Immunoprecipitations with hTAP1 and hTAP2 (A) or hTAP1 alone (B) were separated by SDS–PAGE. The 10–15% gradient gel was exposed to X-ray film for 7 days. Identical amounts of lysates were present in each lane of (A) and (B) as revealed by Coomassie blue staining (not shown). The protein band corresponding to TAP1 in (B) was scanned. (**C**) Percentage of the TAP1 labeling intensity obtained in the absence of His-ICP47 depicted graphically as a function of the concentration of His-ICP47.

which allows direct measurement of peptide binding to microsomes (Van Endert *et al.*, 1994; Uebel *et al.*, 1995). As reporter peptide we used peptide RRYNASTEL (R-9-L) which has been shown to bind with high affinity to human

Table II. Relative efficiencies of peptides in competition for binding to TAP1/TAP2 microsomes

Competitor	Sequence	IC_{50}[a]	
		Human TAP	Mouse TAP
R-9-L	RRYNASTEL	1.3 ± 0.2	1.5 ± 0.4
R-10-A	RYWANATRSA	4.5 ± 0.1	29.0 ± 1.4
R-10-E	RYWANATRSE	>1000	>1000
R-10-F	RYWANATRSF	1.9 ± 0.7	0.6 ± 0.3
R-10-K	RYWANATRSK	23.0 ± 4.2	>1000
R-10-Q	RYWANATRSQ	3.5 ± 1.4	12.5 ± 0.7
R-10-T	RYWANATRST	33.5 ± 17.7	24.0 ± 5.6

[a]Molar excess required for 50% inhibition of specific binding of R-9-L (300 nM).

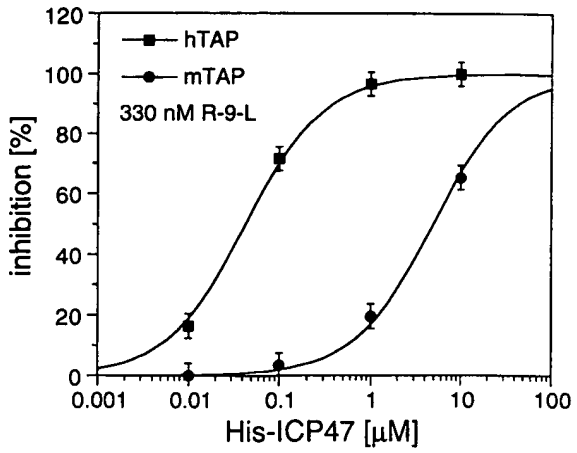

Fig. 7. Inhibition of peptide binding to human or murine TAP microsomes by His-ICP47. Peptide binding of radiolabeled peptide (330 nM RRYNASTEL, R-9-L) to microsomes containing human TAP (■) or murine TAP (●) was studied at various concentrations of His-ICP47. The concentration needed for 50% inhibition of reporter peptide binding (IC_{50}) was calculated from the competitor curve as described by Uebel *et al.* (1995). 100% binding corresponds to 70 000 ± 1400 c.p.m. The concentration of ICP47 needed for 50% inhibition was 42 ± 2 nM for human TAP and 5.0 ± 0.3 μM for murine TAP. All results were obtained by duplicate or triplicate measurements. Error bars indicate SD.

TAP expressed in insect cells (K_d = 310 nM; Uebel *et al.*, 1995). Similarly, Scatchard analysis revealed a K_d of 410 nM for R-9-L binding to murine TAP expressed in SC2 cells (data not shown). Furthermore, similar concentrations of competitor peptide (unlabeled R-9-L) were required for half-maximal inhibition of R-9-L binding to both human and murine TAP present in insect cell microsomes (Table II).

Binding of R-9-L, which was added at 330 nM corresponding to ~50% saturation of binding sites, was inhibited by His-ICP47 at a half-maximal concentration of 42 nM for human TAP (Figure 7). This efficient inhibition of peptide binding to human TAP-expressing insect cells is consistent with the inhibition of peptide crosslinking to human microsomes shown in Figure 6. In both assays, half-maximal inhibition of peptide binding by His-ICP47 was achieved at concentrations approximately one order of magnitude below the reporter peptide concentration. Thus, comparison with the affinity constants measured for peptide binding (300–400 nM) suggests that ICP47 binds to human TAP with a K_d in the region of 50 nM. Most peptides bind to TAP with lower affinities than the reporter peptide R-9-L used in these experiments (Van Endert *et al.*, 1994; Uebel *et al.*, 1995), suggesting that ICP47 binds with orders of magnitude higher affinity to human TAP than the majority of peptides.

By contrast to human TAP, half-maximal inhibition of R-9-L binding to insect cell microsomes containing murine TAP required a 15-fold molar excess over the reporter peptide concentration (Figure 7), which indicates that His-ICP47 displays a 100-fold difference in affinity between human and murine TAP. We conclude that ICP47 is unable to block peptide translocation by murine TAP (Figure 4) as a consequence of this difference in affinity.

This species-specific TAP binding of ICP47 could be caused by structural differences in the substrate-binding site of murine and human TAP molecules. However, it is not known whether human and murine TAP differ with respect to their affinity to different peptides, although ample evidence exists that they transport different sets of peptides (Momburg *et al.*, 1994b; Schumacher *et al.*, 1994). Therefore we compared the previously described peptide series R-10-X (Momburg *et al.*, 1994b) with respect to the ability of individual peptides to compete for R-9-L binding to human and murine TAP-containing insect cell microsomes (Table II). For both species, we obtained similar IC_{50} values for peptides R-9-L, R-10-F and R-10-T. Interestingly, R-10-E was unable to compete for R-9-L binding to either TAP species. Peptides R-10-A and R-10-Q seem to bind with a somewhat higher affinity to human than to mouse TAP. However, a pronounced difference between the two species was only evident for R-10-K binding, indicating that, indeed, there are structural differences between the peptide-binding site of human and murine TAP molecules. It seems likely that differences in substrate-binding affinity determine the observed species specificity of peptide transport. Like R-10-K, ICP47 might be able to bind to the peptide-binding site of human TAP but not to that of murine TAP.

Discussion

To study the molecular mechanism by which ICP47 prevents TAP-mediated translocation of peptides across the ER membrane, we have monitored the binding of either ATP or peptide substrates to TAP in the presence of ICP47. Neither *in vivo*-induced ICP47 nor added purified ICP47 was able to prevent photoaffinity labeling by azido-ATP of the ATP-binding sites within TAP1 or TAP2. Thus, ICP47 does not seem to act by preventing ATP binding. By contrast, photoaffinity labeling of the substrate-binding site by a photoactivatable peptide derivative was completely blocked by ICP47 which was either induced before microsome preparation or which was added to the microsomes before photocrosslinking. We conclude that ICP47 inhibits peptide translocation by preventing peptides from binding to TAP, which represents the first of several individual steps occurring during the translocation of peptides.

This conclusion was further supported by our finding that peptides did not bind to microsomes isolated from human TAP-overexpressing insect cells in the presence of ICP47 and consequently peptides were not transported. Since insect cell-expressed TAP is inhibited by ICP47,

this result renders it unlikely that specialized proteins other than ICP47 and TAP are involved in this process. Interestingly, the concentration of ICP47 required for half-maximal inhibition of peptide binding to human TAP was several fold lower compared with the IC_{50} measured in the peptide transport assay (compare Figures 4 and 7). This result is in line with the observation that peptides which cannot be glycosylated inside the ER compete less efficiently for transport than for binding (Van Endert *et al.*, 1994). The peptide binding assay measures a reversible association of peptides with TAP whereas several steps including irreversible glycosylation of peptides, which might be limiting the overall process, are assayed during the peptide transport assay. The IC_{50} values of competitor peptides containing a glycosylation motif are the summary of competition at the level of glycosylation and at the level of TAP binding. As a result such peptides might be better competitors for transport than for binding compared with peptides lacking a glycosylation signal (Van Endert *et al.*, 1994). Similarly, ICP47 is unable to compete for glycosylation, unlike the competitor peptides R-10-T and R-8-L used for comparison. Since ICP47 was as effective in inhibiting peptide transport as R-10-T or R-8-L (Table I) we conclude that, with respect to TAP binding, the affinity of ICP47 for TAP has in fact to be higher than that of the two peptides. Consistently, ICP47 competed more efficiently for TAP binding than the high-affinity peptide R-9-L (Figure 7 and Table II). In summary, both peptide transport and peptide binding assays suggested that ICP47 binds with higher affinity to TAP than peptides.

Taken together with the previous observation that ICP47 co-precipitates with the TAP heterodimer (Früh *et al.*, 1995; Hill *et al.*, 1995), these findings suggest that ICP47 prevents substrates from binding to TAP by associating with the TAP molecule. Since inhibition of peptide binding by ICP47 follows the same concentration-dependent and saturable function as observed for competitor peptides, it seems that ICP47 interacts with the peptide-binding site of TAP. This hypothesis is also supported by our finding that both ICP47 and peptides bind species-specifically to TAP. However, Scatchard analysis of peptide binding in the presence of different His-ICP47 concentrations revealed both competitive and non-competitive inhibition, depending on whether His-ICP47 was added simultaneously with peptides or preincubated with TAP (data not shown). Thus, it can be assumed that ICP47 interacts with additional residues of TAP compared with peptides, which is also supported by our observation that ICP47 displayed a much higher affinity for TAP than most peptides.

Little is known about the structure of the peptide-binding site except that both TAP subunits are part of it, because both TAP1 and TAP2 are found to be crosslinked to peptide (Androlewicz *et al.*, 1994). Our data suggest that the structure of this binding site is not conserved between human and murine TAP, since we observed a much lower affinity of R-10-K for binding to murine TAP than to human TAP. This finding is consistent with previous results showing that murine TAP is less efficient than human TAP in translocating peptides with basic carboxy-termini (Momburg *et al.*, 1994b; Schumacher *et al.*, 1994). In agreement with previous observations made in murine cells (Schumacher *et al.*, 1994) but contrary to results from human cells (Momburg *et al.*, 1994b), peptides with acidic carboxy-termini did not bind to either human or murine TAP, whereas all other peptides tested showed measurable affinities. Although some human class I MHC molecules bind peptides with basic carboxy-termini, this has not been observed for murine class I MHC molecules (Rammensee *et al.*, 1993). Moreover, neither human nor murine class I MHC molecules bind peptides with acidic carboxy-termini. Thus, the species-specific peptide-binding characteristics described here seem to correlate better with the known binding motifs of human and murine class I MHC molecules than with those of previous data obtained by measuring peptide translocation. Since our data result from a very limited set of peptides, it will be interesting to compare the peptide-binding characteristics of TAP from different species with a larger set or a library of peptides. Furthermore, the species-specific peptide and ICP47 binding demonstrated here should be useful to map some of the residues on TAP contributing to this binding site.

In comparison with peptides we estimate that ICP47 binds with an affinity of 50 nM to human and 5 μM to mouse TAP. This 100-fold lower affinity of ICP47 for murine TAP causes insufficient blocking of murine TAP *in vivo*, since it has been observed that HSV-1-infected murine cells do not show a marked reduction in surface expression of class I MHC molecules (Jennings *et al.*, 1985) and antigen presentation is not blocked by HSV-1 in mouse fibroblasts (York *et al.*, 1994). Moreover, transfection of ICP47-expressing HeLa cells with murine TAP restores class I MHC surface expression (Früh *et al.*, 1995). Thus, it seems that similar to our *in vitro* results, ICP47 does not achieve the concentrations which would be necessary to block TAP within murine cells. This low affinity of ICP47 for murine TAP should be taken into consideration when using rodents as models to study the immune response against HSV-1. In fact, many differences are apparent between the course of HSV-1 infections in humans and rodents (reviewed in Mester and Rouse, 1991). Whereas in some mouse models $CD8^+$ T-cell responses dominate, it has been suggested that in humans the predominant immune protection is afforded by natural killer cells (Brutkiewicz and Welsh, 1995). It might be that the species-specific activity of ICP47 is crucially involved in stimulating different immune effector cells. The inability of ICP47 to prevent peptide loading onto class I MHC molecules in mice would lead to activation of virus-specific $CD8^+$ T-cells. By contrast, such CTL activation might be blocked in humans due to the activity of ICP47. Since natural killer cells specifically lyse cells which lack surface expression of class I MHC molecules (Leibson, 1995), ICP47 might help herpes simplex virus to escape one line of immune defense in humans, but by doing so it might activate another.

Material and methods

DNA constructs

For bacterial expression, a hexahistidine tag (MHHHHHHIEGR) was fused to the amino-terminus of ICP47 by polymerase chain reaction (PCR) using primers containing the appropriate codons and restriction sites. The resulting fragment was cloned into the *NdeI* and *BamHI* restriction sites of pRK171 (Früh *et al.*, 1992).

Murine TAP1 and TAP2 were obtained from RMA cells by PCR as

described (Yang *et al.*, 1992). mTAP1 was cloned as a *SacI–SalI* fragment into pRMHa.3 (Jackson *et al.*, 1992). A hexahistidine tag was fused to the carboxy-terminus of mTAP1 by PCR using a 3' primer which contained the appropriate codons. No difference in transport activity to wild-type TAP1 was observed for the histidine-tagged construct (not shown). TAP2 was cloned as *NcoI–SalI* fragment into pRMHa.3. Cloning of human TAP1-His and human TAP2 and expression in baculovirus were as described previously (Meyer *et al.*, 1994).

Expression and purification of His-ICP47

The bacterial strain BLys (Novagen), containing T7 polymerase and T7 lysozyme, was transformed with His-ICP47.pRK171 and grown in the presence of 36 μg/ml tetracycline and 100 μg/ml ampicillin. 0.5 l were inoculated with 5 ml of a fresh overnight culture and expression was induced with 1 mM IPTG (Sigma) at an OD_{600} of 1.0. After 2–4 h cells were harvested by centrifugation. The cell pellet was solubilized in 50 ml 6 M GuHCl, 0.1 M sodium phosphate pH 8.0 by stirring for 16 h at 4°C. Insoluble material was removed by centrifugation for 15 min at 15 000 *g*. To the supernatant was added 2 ml of packed Ni-NTA–agarose (Qiagen) and the suspension was rotated for 2 h at 25°C. Agarose beads were collected by centrifugation (900 *g*×2 min) and washed five times with 50 ml of 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl pH 8.0. Subsequently, the beads were packed into a column and His-ICP47 was eluted by stepwise lowering of the pH to 4.0. Fractions containing His-ICP47 were pooled and further purified by reverse-phase HPLC using a 20–70% acetonitrile gradient and 0.05% trifluoroacetic acid. Peak fractions containing His-ICP47 were lyophilized. Purified His-ICP47 was resuspended in water before use.

Cell lines and cell culture

The generation of recombinant baculoviruses expressing human TAP1-His and hTAP2 has been described previously (Meyer *et al.*, 1994). Sf9 (*Spodoptera frugiperda*) cells were grown as a monolayer according to standard procedures in TC100 insect cell medium with 10% fetal calf serum and infection was routinely performed with a m.o.i. of 3–5.

ICP-O20 cells, a stable HeLa cell line in which ICP47 expression can be up-regulated by the removal of tetracycline, were grown in the presence (1.0 μg/ml) or in the absence of tetracycline as described (Früh *et al.*, 1995).

Drosophila melanogaster SC2 cells were transfected with mTAP1-His.pRMHa3, mTAP2.pRMHa3 hCD8 and the neo-resistance plasmid phshsneo (Jackson *et al.*, 1992) in a ratio of pRMHa3 11:11:1:1. G418-resistant cells were selected by growing the cells with 0.5 mg/ml of G418 (Gibco) in Schneider medium (Life Technologies, Inc) containing 10% fetal calf serum.

Preparation of microsomes

Sf9 cells were harvested 60–72 h post-infection with hTAP-containing baculovirus. For induction of mTAP expression in *D.melanogaster* cells, $CuSO_4$ (1 mM) was added to the medium 16 h before harvesting the cells. ICP-O20 cells were induced with IFN-γ for 48 h and either grown with or without 1 μg/ml tetracycline during the same time period. Cells were harvested by centrifugation, washed once with PBS and lysed after resuspension in cavitation buffer (250 mM sucrose, 25 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM calcium acetate, 50 mM Tris–HCl pH 7.4) with proteinase inhibitor mix at 10^8 cells/ml by repeated drawing through a 26-gauge needle. Nuclei and non-lysed cells were removed by centrifugation (300 *g*×5 min at 4°C) and the supernatant was diluted 6.4-fold with 2.5 M sucrose in gradient buffer (150 mM potassium acetate, 5 mM magnesium acetate, 50 mM Tris–HCl pH 7.4) and stepwise overlaid with 2.0 M and 1.3 M sucrose in gradient buffer and with cavitation buffer. After centrifugation overnight (104 000 *g* at 4°C) the turbid fraction at the interface of the 2.0 and 1.3 M sucrose solution was collected, diluted 2-fold with PBS/1 mM DTT and centrifuged (227 000 *g*×1 h at 4°C). The microsomes were resuspended in PBS/1 mM DTT, snap-frozen in liquid nitrogen and stored at –80°C.

Antibodies and antisera

Monoclonal antibodies 148.3 (anti-hTAP1) and 429.4 (anti-hTAP2) have been described in Meyer *et al.* (1994) and Van Endert *et al.* (1994), respectively. Generation of polyclonal anti-mTAP1 antisera (1:500) and anti-mTAP2 antisera was described in Früh *et al.* (1992) and Wang *et al.* (1994), respectively.

Immunoblot and immunoprecipitation

Microsomes containing human or murine TAP (~5 μg of total protein) were separated on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Immunoblot analysis was done using ECL (Amersham) with the anti-human TAP monoclonal antibodies (hybridoma supernatant, diluted 1:10), anti-murine TAP antisera (diluted 1:500) or anti-histidine tag purified monoclonal antibody (diluted 1:15; Dianova).

Immunoprecipitations from ICP-O20 cells were performed as described by Früh *et al.* (1995). Immunoprecipitated material was separated by SDS–PAGE. Gels were dried and exposed to X-ray films (Amersham).

Peptide transport assays

For competition assays, microsomes prepared from insect cells (50 μg of total protein) were incubated with competitor peptides or His-ICP47 for 5 min at 37°C before adding 250 nM reporter peptides in 100 μl transport buffer (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 0.1% dialyzed BSA, 1 mM DTT pH 7.3) as described previously (Meyer *et al.*, 1994). After incubation for 2.5 min at 37°C, the microsomes were washed and lysed in 1 ml lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40 pH 7.5. The lysate was incubated with 100 μl concanavalin A (ConA)–Sepharose (Sigma) for 1.5 h at 4°C, washed twice and the bound peptide was eluted with 1 ml of 200 mM α-methylmannoside (Sigma) in lysis buffer (1 h at room temperature) and quantified by gamma-counting.

Non-competitive peptide transport assays (Neefjes *et al.*, 1993) with different peptides and insect cell microsomes were carried out using 600 nM labeled peptides and 20 min incubation at 37°C. Incubation with ConA–Sepharose was for 16 h at 4°C; bound radioactivity was counted after five washes. TAP-dependent peptide translocation in streptolysin O (Murex)-permeabilized HeLa cells was assayed as described in Früh *et al.* (1995).

Photocrosslinking of peptides and ATP

Peptide KYWANATRSGL was conjugated to the photoreactive crosslinker *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB, Pierce) and iodinated using chloramine T. 50 μl of microsomes were added to 300 μl assay buffer (PBS containing 1 mg/ml dialyzed bovine serum albumin, 1 mM DTT and 2 mM $MgCl_2$). The membrane suspensions were transferred to a 6-well dish (Corning), and 2.5 μM iodinated peptide was added. Photocrosslinking was initiated by irradiation with a 254 nm UV lamp (intensity, 6200 $\mu W/cm^2$). After 10 min of irradiation, the reaction was quenched by addition of lysis buffer (1% NP40 in PBS). His-ICP47 was preincubated with microsomes for 10 min at 37°C before iodinated peptide was added.

ATP photoaffinity labeling was performed as described (Müller *et al.*, 1994; Wang *et al.*, 1994). Reactions were carried out in 500 ml ice-cold labeling buffer (100 mM Tris–HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 0.5 μg/ml leupeptin) containing 100 μl of microsomes, various concentration of [α-^{32}P]8-azido-ATP (Amersham) and, when appropriate, cold ATP as competitor.

Peptide binding and competition assays

Microsomes were diluted with assay buffer (PBS with 1 mg/ml dialyzed bovine serum albumin, 1 mM DTT, 2 mM $MgCl_2$) to a final protein concentration of 25–50 μg/ml. The protein concentration was determined using a modified Lowry assay. This suspension was homogenized by drawing through a 23-gauge needle. From this suspension, 150 μl were incubated with 330 nM of the radiolabeled peptide R-9-L and the appropriate amount of unlabeled competitor for 8 min at 4°C. Thereafter, 350 μl ice-cold assay buffer was added and the microsomes were pelleted by centrifugation (12 000 *g*×10 min at 4°C). Vesicle-associated radioactivity was quantified by gamma-counting after one washing step with 500 μl of ice-cold assay buffer. The amount of bound peptide was corrected by non-specific binding of baculovirus microsomes. The data set was fitted by the competition function as described by Uebel *et al.* (1995). The concentration needed for 50% inhibition was determined from two or three competition assays.

Acknowledgements

We thank M.Squier for help with graphics and G.Hämmerling for providing R-10-X peptides. The support by the peptide and DNA core facilities of the R.W.Johnson Pharmaceutical Research Institute and the Max-Planck-Institut für Biochemie is also gratefully acknowledged. R.T. is supported by grants SFB266-D11 and Ta157/2 from the Deutsche Forschungsgemeinschaft.

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Received on December 14, 1995; revised on March 4, 1996

Restoration of the Expression of Transporters Associated with Antigen Processing in Lung Carcinoma Increases Tumor-Specific Immune Responses and Survival

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Abstract

A wide variety of human carcinomas have low expression of tumor-associated antigen presentation in the context of MHC class I antigens due to defects in the antigen presentation pathway. This immune evasion mechanism renders many tumors unrecognizable by host immune surveillance mechanisms. The present study examines the expression of HLA, tapasin, transporter associated with antigen processing 1 (TAP1), and β2 microglobulin in human small cell lung carcinoma and non–small cell lung carcinoma. Immunohistochemical staining showed severe impairment of the antigen presentation pathway in all patients. In order to recover tumor immunogenicity, a nonreplicating adenovirus expressing human TAP1 (AdhTAP1) was used to restore the expression of TAP1 in the antigen presentation pathway–deficient mouse lung carcinoma cell line, CMT.64. Infection of CMT.64 cells with AdhTAP1 increased MHC class I antigen surface expression, antigen presentation, and susceptibility to antigen-specific CTLs. Fluorescence-activated cell sorting and ELISPOT analysis showed that AdhTAP1 treatment significantly increased dendritic cell cross-presentation and cross-priming of tumor antigens. Furthermore, *ex vivo* and *in vivo* AdhTAP1 treatment significantly retarded tumor growth and increased survival of mice bearing CMT.64 tumors. Fluorescence-activated cell sorting analysis and immunohistochemical staining showed a significant increase in $CD8^+$ and $CD4^+$ T cells and $CD11c^+$ dendritic cells infiltrating the tumors. The results show that TAP should be considered as a part of the immunotherapies for various cancers because it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic composition of the tumor or the MHC haplotypes of the host. (Cancer Res 2005; 65(17): 7926-33)

Introduction

The discovery of HLA class I restricted, tumor-associated, and tumor-specific antigens has reinvigorated the clinical development of immunotherapies for the treatment of carcinomas. The appeal of immunotherapies is the potential to control disseminated, metastatic disease with a minimum of toxic side effects due to the immune system's exquisite specificity. Many of the therapies involve vaccination with protein/peptide antigens, plasmids, or recombinant virus–encoding genes for antigens, or whole cell vaccines which consist of irradiated autologous or allogeneic tumor cells, or autologous dendritic cells with a variety of modifications (1). These varied approaches aim to stimulate a T cell–mediated antitumor immune response and are based on the hypothesis that the cellular arm of the immune system can control neoplastic disease through immunosurveillance and targeting of tumor-specific antigens. Although conceptually appealing, the success of cancer vaccines and immunotherapies in humans is variable. In most cases, the vaccines are very well tolerated and specific immune responses to particular antigens are made but the response rate of the disease to the therapy is low. The reason for the low response rates are thought to be due to several factors which include low immunogenicity and tolerance to tumor-associated antigens (TAA), immunosuppressive microenvironments in the tumor and antigen loss variants arising in the tumor through immunoselection (2, 3). The antigen loss variants are characterized by the absence or decreased expression of MHC class I antigens on the tumor cell surface. The alterations in the expression of MHC molecules play a crucial step in tumor development due to the role of MHC antigens in antigen presentation to T lymphocytes and the regulation of natural killer cell function. In some cases, the rates of total HLA class I antigen losses are close to 100% (4, 5). These MHC losses can be produced at any step required for HLA synthesis, assembly, transport, or expression on the cell surface. In humans, the loss of HLA expression in a wide variety of carcinomas is often associated with transporter associated with antigen processing (TAP1 and TAP2) down-regulation (6), which is strongly correlated with disease progression and metastasis in patients (7). Loss of TAP activity leads to failure to transport peptides from the cytoplasm to the lumen of the endoplasmic reticulum, and hence, failure of the class I antigen processing pathway (APP). For example, the Buf1280 cell line from a metastatic lesion of a melanoma patient is TAP1-deficient due to a base pair deletion at position 1,489 of the *TAP1* gene, which resulted in no *TAP1* gene expression. The impaired TAP1 protein expression results in deficiencies in TAP2 protein expression, peptide binding, translocation, and MHC class I antigen surface expression. Stable *TAP1* gene transfer reconstitutes the described defects (8). This phenomenon has also been observed in a human small cell carcinoma cell line deficient for TAP1 and low molecular weight protein 2 (LMP2). Restoration of TAP1 expression alone increased HLA surface expression but restoration of LMP2 alone did not complement surface expression of HLA molecules (9). The mouse lung carcinoma, CMT.64, a cell line derived from a

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doi:10.1158/0008-5472.CAN-04-3977

spontaneously occurring lung carcinoma in a C57BL/6 mouse (10), is characterized by down-regulation of many of the components of the APP. These components include MHC class I heavy chain, β2 microglobulin (β_2-m), proteasome subunits (LMP2 and LMP7), TAP1 and 2 (11, 12) and tapasin.[5] Despite these multiple defects, restoration of TAP1 itself leads to partial restoration of MHC class I antigen surface expression (11). Studies in C57BL/6 mice show that the introduction of mixtures of TAP1-positive and TAP1-negative cells produced tumors composed exclusively of TAP1-negative cells, indicating selection and evasion of immune surveillance in cells with the TAP deficiency (13). Mice bearing TAP-deficient CMT.64 tumors are able to clear the tumor load after treatment with replicating vaccinia virus encoding rat TAP1 alone and develop protective immunity upon subsequent challenge with CMT.64 cells (14). In the light of these studies, *TAP* gene transfer has a number of potential advantages as an immunotherapeutic strategy for the treatment of cancer. Restoration of TAP activity could restore the immunogenicity of the tumor by restoring the presentation of a number of tumor-specific antigens allowing an antitumor immune response that is HLA-specific, tumor-specific, and patient-specific (14).

In this study, we examine the expression of cell surface HLA and components of the APP, which include β_2-m, TAP1, and tapasin, in lung carcinomas from patients that have undergone lung resection. We make a replication-deficient ($E1^-/E3^-$) adenovirus vector expressing the human TAP1 (AdhTAP1) that can restore antigen processing and MHC class I antigen surface expression in the CMT.64 carcinoma cell line. We treat mice bearing CMT.64 tumors with AdhTAP1, to ascertain if boosting immune responses are capable of controlling the progression of the disease *in vivo*.

Materials and Methods

Antigen presentation pathway status in human lung carcinoma. Paraffin- embedded sections of human small cell lung carcinoma (SCLC, $n = 9$) and non–small cell lung carcinoma (NSCLC, $n = 10$) tumors were obtained from the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, St Paul's Hospital, University of British Columbia, Vancouver, BC. The Centre maintains a registry of human lung and tumor samples cross-referenced with relevant clinical information (15, 16). The procedures used to obtain the tissue for the registry were approved by the Institutional Review Board of the University of British Columbia. Lung parenchyma adjacent to the tumor was used as a control for each case. Tumor sections were stained with antibodies directed against HLA class I heavy chain (monoclonal antibody HC-10 recognizing an epitope on β_2-m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, and -HLAA33 heavy chains as well as on all β_2-m-free HLA-B heavy chains; ref. 17), β_2-m (mouse anti-β_2-m monoclonal antibody purchased from Dako, Hamburg, Germany), TAP1 (mouse anti-TAP1 monoclonal antibody 148.3), and tapasin (monoclonal antibody TO-3 was generated from a BALB/c mouse immunized with a tapasin-derived peptide and fusion protein; ref. 17). Staining was developed with the standard streptavidin-biotin-immunoperoxidase technique [LSAB 2 system-horseradish peroxidase (HRP), Dako]. The percentage of stained tumor cells was determined in each lesion and classified according to the HLA workshop criteria as: (0, negative): <25% of stained tumor cells; (1, heterogeneous): >25 to <75% of stained tumor cells; and (2, positive): >75% of stained tumor cells. Negative controls were done by omitting primary antibodies (17).

Mice, cells, and viruses. C57BL/6 ($H\text{-}2^b$) mice, 6 to 8 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed and bred at the Biotechnology Breeding Facility, University of British Columbia. HEK 293 cells (American Type Culture Collection, Rockville, MD), CRE8 cells (18), CMT.64 cells (10), T1 (ATCC, CRL-1991, a TAP1-positive cell line), and T2 cells (ATCC, CRL-1992, a TAP1-negative cell line) were cultured in DMEM supplemented with 10% fetal bovine serum except T2 cells, which received 20% fetal bovine serum, 2 mmol/L L-glutamine, streptomycin (0.1 mg/mL), and penicillin (100 units/mL). All recombinant adenoviruses were propagated and titrated in HEK 293 cells. Splenocytes were cultured in RPMI 1640, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, 50 μmol/L β-mercaptoethanol, 1 mmol/L sodium pyruvate, 0.1 mmol/L essential amino acids, and 10% fetal bovine serum.

Construction of recombinant adenovirus vectors. Human *TAP1* was amplified from pCEP/*hTAP1* (provided by Dr. P. Wang, England) with the following primers, 5′-CAT AGC ATG CAT GGC TAG CTC TAG GTG TCC C-3′, which introduced the *Sph*I site, and 5′-GCA ATC TAG ATC ATT CTG GAG CAT CTG CAG G-3′, which introduced the *Xba*I site. The *hTAP1* PCR fragment was digested with restriction enzymes *Sph*I and *Xba*I, and ligated with T4 DNA ligase to the *Sph*I and *Xba*I sites of padlox (18), a plasmid shuttle vector for making AdhTAP1. Padlox/*hTAP1* was isolated and sequenced to ensure sequence fidelity. Pad/*hTAP1*, linearized with *Sfi*I, was cotransfected along with Ψ5 DNA (Ψ5 is an E1 and E3 deleted version of Ad5 containing loxP sites flanking the packaging site) into CRE8 cells using a modified LipofectAMINE protocol (Invitrogen Life Technologies, Carlsbad, CA; ref. 18). After development of confluent cytopathic effect (7 or 8 days), cells were freeze/thawed thrice and the resultant lysate passaged thrice in CRE8 cells. Plaques were screened by immunofluorescence assay for the presence of hTAP1. AdhTAP1 was plaque-purified and propagated in HEK 293 cells followed by purification and concentration by CsCl centrifugation. Purified virus was dialyzed against 10% glycerol in PBS (pH 7.4) and stored at −80°C. AdhTAP1 was confirmed by PCR and DNA sequencing using *hTAP1*-specific primers and primers specific to adenovirus sequences flanking either side of the *hTAP1* cDNA (data not shown). Infectious virus titer was determined on HEK 293 cells by plaque assay and absorbance at 260 nm wavelength of light was used to determine viral particle number (19). Typical particle/plaque-forming units (PFU) ratios were 100. Virus multiplicity of infection (MOI) was defined as PFU/cell.

hTAP1 expression in CMT.64 cells. To examine the time course of hTAP expression, CMT.64 cells were infected with AdhTAP1 or Ψ5 at 10 MOI and harvested every day for 7 days. To examine hTAP1 response to increasing doses of AdhTAP1, CMT.64 cells were infected with AdhTAP1 or Ψ5 at 50, 10, 2, 0.4, 0.08 MOI and harvested 2 days after infection. Both time course and dose response studies were analyzed by SDS-PAGE followed by Western blot. The samples were reacted with rabbit anti-hTAP1 antibodies (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) and mouse monoclonal anti-human β-actin antibody (Sigma-Aldrich, Oakville, Ontario, Canada). Goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP (Jackson ImmunoResearch Lab, West Grove, PA) were used as secondary antibodies. The bands were visualized by exposure to Hyperfilm (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) using the enhanced chemiluminescence procedure.

Surface expression of MHC class I. CMT.64 cells were infected with AdhTAP1 or Ψ5 at 20 MOI. Two days after infection, the cells were incubated with anti-MHC class I monoclonal antibodies, y3 ($H\text{-}2K^b$-specific) and 28.14.8S ($H\text{-}2D^b$-specific) at 4°C for 30 minutes (14). Bound antibodies were detected by goat anti-mouse IgG-FITC (Jackson ImmunoResearch Lab). Fluorescence-activated cell sorting (FACS) analysis was done in a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

CTL assay. Cytotoxicity assay was measured in a standard 4 hour ^{51}Cr-release assay. In brief, we established a stable cell line, CMT/VSV-Np, which was CMT.64 cells expressing an $H\text{-}2K^b$ restricted immunodominant determinant from vesicular stomatitis virus (VSV) nucleocapsid protein (amino acids 52-59). CMT/VSV-Np cells were infected with AdhTAP1 or Ψ5 at 30 MOI for 1 day followed by ^{51}Cr (Amersham, Arlington Heights, IL) label. VSV-specific CTL effectors were generated by i.p. injection of mice with 5×10^7 PFU VSV. Splenocytes were collected 5 days after infection and cultured in RPMI 1640 complete medium plus 1 μmol/L VSV-Np (52-59; Peptide Synthesis Lab., University of British Columbia) for 5 days. The percentage of killed cells was calculated using the formula: % release = 100

[5] Unpublished data.

× (cpm experiment − cpm spontaneous release) / (cpm maximum release − cpm spontaneous release).

ELISPOT analysis of tumor-associated antigen–specific INF-γ–secreting splenocytes. CMT.64 cells (6×10^6 cells) were incubated with AdhTAP1 or Ψ5 (25 MOI) or PBS at 37°C for 2 hours followed by irradiation (10,000 rad for 30 minutes). Mice were immunized by three separate i.p. injections of 2×10^6 treated cells, with each injection separated by a 7-day interval. Nine days after the last immunization, splenocytes were isolated and cultured *in vitro* in RPMI 1640 complete medium with CMT.64 tumor-associated antigen (TAA) MUT1 or MUT2 (20 μg/mL peptide) for 14 hours. The CMT.64 TAA, MUT1 (FEQNTAQP), and MUT2 (FEQNTAQA; ref. 20), were made by the Peptide Synthesis Lab. The frequency of MUT-specific IFN-γ secreting cells was determined using an ELISPOT assay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cross-presentation of ovalbumin by dendritic cells. Splenic dendritic cells were isolated using CD11c magnetic beads (Miltenyi Biotech, Auburn, CA) and infected with either 20 MOI of AdhTAP1 or Ψ5 for 2 hours, followed by incubation with ovalbumin 5 mg/mL for 16 hours. After incubation, dendritic cells were washed and Fc receptors were blocked (2.4G2 FcγIII/II blocker, BD PharMingen, Mississauga, ON, Canada). Cells were stained with monoclonal antibody 25.D1.16, specific for H-2K^b/SIINFEKL (ovalbumin 257-264) complexes (21, 22), or IgG_1 isotype control antibody followed by phycoerythrin-conjugated rat anti-mouse IgG_1 (BD PharMingen). Surface expression of H-2K^b/SIINFEKL complexes and total H-2K^b were measured by FACS analysis.

Survival studies in mice. For *ex vivo* studies, CMT.64 cells were incubated with AdhTAP1 or Ψ5 at 50, 10, and 2 MOI for 2 hours followed by washing in PBS. Infected cells (4×10^5) were i.p. injected into mice (10 mice/group) and survival was followed for 90 days. Aliquots of *ex vivo* infected cells were cultured for 1 week to confirm viability. Mice were killed upon signs of morbidity and this time was recorded as death. For *in vivo* treatments, tumors were established in three groups of 24 to 28 mice per group by i.p. injection using 4×10^5 CMT.64 cells in 500 μL PBS/mouse. On days 1, 3, 5, and 8 after the introduction of CMT.64 cells, mice were injected i.p. with AdhTAP1, Ψ5, or PBS using 1×10^8 PFU/mouse/injection in 500 μL PBS, and mouse survival was followed for 90 days. During the experiment, four to eight mice were killed from each group at selected times to observe tumor growth pattern and to measure the number of tumor-infiltrating CD4$^+$ and CD8$^+$ T lymphocytes and CD11c$^+$ dendritic cells.

Tumor-infiltrating lymphocytes and dendritic cells. For detection of tumor-infiltrating lymphocytes subsets CD4$^+$ and CD8$^+$ T cells by FACS, tumors were washed and homogenized into single-cell suspensions and incubated with FITC-conjugated rat anti-mouse CD8α (Ly-2) monoclonal antibody and R-phycoerythrin-conjugated rat anti-mouse CD4 (L3T4) monoclonal antibody (BD PharMingen). For detection of CD4$^+$ and CD8$^+$ T cells and CD11c$^+$ dendritic cells using immunohistochemical staining, 8-μm frozen sections were acetone-fixed and incubated with the following antibodies: rat anti-mouse CD4 monoclonal antibody (RM4-5), rat anti-mouse CD8 monoclonal antibody (53-6.7), and hamster anti-mouse CD11c (HL3). Rat IgG_{2a} and hamster IgG were used as isotype controls. Antibody binding was detected with biotinylated anti-hamster IgG cocktail secondary antibodies and streptavidin-HRP and a 3,3′-diaminobenzidine detection system. All the reagents were purchased from BD PharMingen.

Statistical analysis. To compare FACS population histograms for the analysis of H-2K^b or H-2K^b/SIINFEKL complexes expressed on dendritic cells infected with AdhTAP1 or Ψ5 (control vector) a comparison algorithm was used called Probability Binning (Multivariate Comparison FlowJo 3.7.1.). This algorithm is related to the Cox χ^2 approach, but with modified binning such that it minimizes the maximal expected variance and has been shown to detect small quantitative differences between two populations (23, 24). To establish biological significance, a cut-off value of $T(X)>10$ was empirically determined and $P < 0.01$ (99% confidence) was considered significant. Kaplan-Meier survival analysis was used to compare the effect of AdhTAP1 treatment on mice bearing CMT.64 tumors. The data were considered statistically different if $P < 0.05$ after the Bonferroni correction for multiple comparisons.

Results

Antigen presentation pathway in patient samples. Almost all tumors were either negative (Fig. 1*A*) or showed heterogeneous patterns of expression for components of the APP. Only one lesion was homogeneously positive for the TAP1 protein (Fig. 1*B*) and two lesions homogeneously positive expressed tapasin. In contrast, the adjacent lung parenchyma for all cases stained homogeneously positive for components of APP (Fig. 1*C*). Neither of the TAP1- or tapasin-positive lesions showed positive staining for MHC I or β_2-m. The grading for each case is summarized in Table 1. Both SCLC and NSCLC were severely limited in the

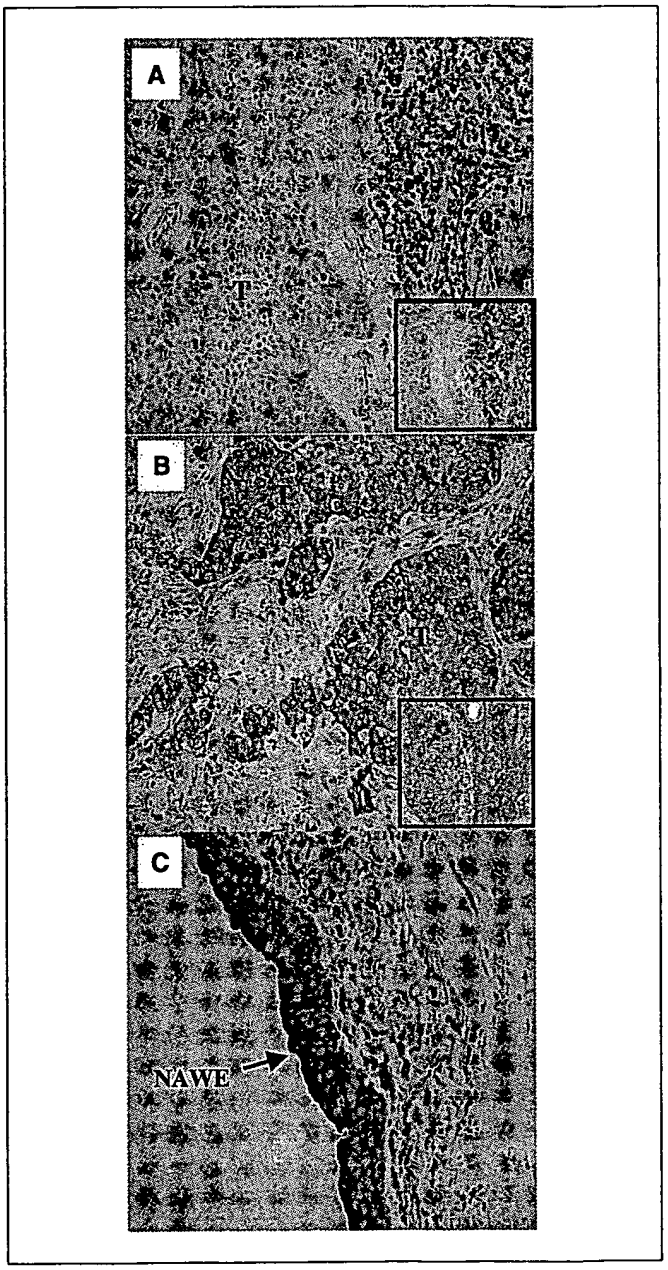

Figure 1. Sample micrographs of immunohistochemical staining for TAP1 in human lung show: (*A*) loss of TAP1 expression in primary lung cancers (grade 0, 400×; inset 200×); (*B*) tumor displaying positive staining for TAP1 (grade 2, 400×; inset 200×); and (*C*) TAP1 staining in normal respiratory epithelium (400×). T, tumor; NAWE, normal airway epithelium.

Table 1. APP status of human small cell lung carcinoma (SCLC) and non-small cell lung carcinoma

Diagnosis	Age	Sex	Stage, N, T	TAP1	HLA I	β_2-m	Tapasin	Total score
SCLC	51	M	3, 2, 2	0	0	0	0	0
SCLC, poorly differentiated	71	M	1, 0, 2	0	0	0	0	0
SCLC	67	F	3, 1, 2	1	0	0	0	1
SCLC	69	F	1, 0, 1	0	0	1	0	1
SCLC	69	M	1, 0, 1	0	0	0	0	0
SCLC	69	M	1, 1, 1	1	1	0	0	2
SCLC	63	F	ND	0	0	0	0	0
SCLC	72	F	1, 0, 1	1	1	1	1	4
SCLC	48	M	3, ND, ND	1	0	0	1	2
Squamous cell carcinoma	75	M	3, 2, 2	0	0	1	0	1
Poorly differentiated adenosquamous	68	M	2, 1, 2	0	0	0	1	1
Moderately differentiated squamous cell	76	M	3, 2, 2	1	0	1	1	3
Well differentiated squamous cell	79	F	3, 3, 1	1	0	1	1	3
Moderately differentiated squamous cell	71	F	3, ND, ND	0	1	0	0	1
Moderately differentiated squamous cell	70	F	3, ND, ND	2	0	0	2	4
Moderately differentiated squamous cell	58	M	2, ND, 1	0	0	0	1	1
Poorly differentiated squamous cell	55	M	3, 1, 4	1	1	1	0	3
Moderately differentiated squamous cell	74	M	2, ND, ND	0	1	0	0	1
Squamous cell HIV+	61	M	2, ND, ND	0	0	0	2	2

NOTE: Grade 0, <25% cells positive; grade 1, 25% to 75% positive cells; grade 2, >75% positive cells. The maximum total score for each case is 8; ND, not determined.

expression of antigen presentation components, however, no correlation appeared between clinical staging scores and the scores for APP components.

Human TAP1 expression in CMT.64 cells. Western blot analysis showed that CMT.64 cells infected with AdhTAP1 infection resulted in high transgene expression of hTAP1. The expression of hTAP1 lasted at least 7 days and the amount of expression was dose-dependent (Fig. 2*A* and *B*).

AdhTAP1 increases MHC class I surface expression in CMT.64 cells. The effect of hTAP1 expression on cell surface MHC class I antigen expression in AdhTAP1-infected CMT.64 cells was investigated (Fig. 2*C*). FACS analysis showed that normal CMT.64 cells are devoid of surface MHC class I antigen. This is consistent with previous studies demonstrating that multiple APP components are down-regulated in CMT.64 cells, thereby resulting in low surface MHC class I antigen surface expression. Treatment of normal CMT.64 cells with INF-γ causes a very large increase in MHC class I antigen surface expression (11, 12). This shows that APP component expression in CMT.64 cells is inducible by IFN-γ, which leads to the restoration of MHC class I antigens on the cell surface (14). Cell-surface expression of H-2K^b and H-2D^b antigens was increased in CMT.64 cells infected with AdhTAP1 compared with cells infected with Ψ5, which showed no increase in cell surface expression of MHC class I antigens. Therefore, expression of TAP1 alone resulted in restoration of MHC class I surface expression on CMT.64 cells when compared with INF-γ treatment (positive control).

AdhTAP1 restores the antigenicity of CMT.64 cells. A cytotoxicity assay was used to determine if AdhTAP1 enhanced the capability of CMT.64 cells to present antigens. CMT/VSV-Np cells were used as targets for VSV-specific effectors. CMT/VSV-Np cells infected with AdhTAP1 or cells treated with INF-γ (positive control) were sensitive to the cytolytic activity of the VSV-specific effectors, whereas CMT/VSV-Np cells alone or cells infected with Ψ5 (Ad vector control) were resistant to killing (Fig. 2*D*). These results show that hTAP1 expression and activity caused by AdhTAP1 infection can restore sufficient MHC class I restricted antigen presentation of a specific epitope [VSV-Np (52-59)], rendering these cells susceptible to specific cytotoxic activity.

AdhTAP1 increases dendritic cell cross-presentation. The cross-presentation of the H-2K^b restricted ovalbumin epitope, SIINFEKL (ovalbumin 257-264), processed from an exogenous source of ovalbumin, was examined in dendritic cells. The fluorescence specific to H-2K^b/SIINFEKL complexes were measured in dendritic cells infected with AdhTAP1 and compared with dendritic cells infected with Ψ5, both in the presence of ovalbumin. After ovalbumin incubation, mean H-2K^b/SIINFEKL-specific fluorescence was 60% greater in AdhTAP1 infected dendritic cells than in dendritic cells infected with Ψ5 ($P < 0.01$; Fig. 3*A*). In addition to significant increases in MHC class I cross-presentation of exogenous antigens, AdhTAP1 infection also significantly increased the mean fluorescence attributed to total surface H-2K^b by 31% over that seen in Ψ5-infected dendritic cells ($P < 0.01$; Fig. 3*B*).

AdhTAP1 treatment increases tumor-associated antigen–specific INF-γ secreting splenocytes. MUT1- and MUT2-specific cellular immune responses in mice immunized with irradiated AdhTAP1-infected CMT.64 cells were measured using an IFN-γ ELISPOT assay. MUT1 and MUT2 are shared Kb-restricted TAA in CMT.64 cells and another spontaneous C57BL/6 lung carcinoma (3LL). Vaccination with synthetic MUT1 or MUT2 induces CTLs that efficiently kill CMT.64-derived clones, protects mice from CMT.64 metastasis, and affords therapy of established CMT.64 metastases (20). Mice vaccinated with irradiated, AdhTAP1-infected CMT.64 cells showed a large increase in the number of both MUT1- and MUT2-specific, INF-γ-secreting splenocytes compared with mice vaccinated with either irradiated PBS treated cells

or irradiated Ψ5-infected CMT.64 cells. These results indicate that AdhTAP1 treatment of CMT.64 cells induced a Th1-type tumor-specific immune response (Fig. 3*C*).

AdhTAP1 treatment *ex vivo* and *in vivo* increases survival of mice bearing CMT.64 tumors. We examined if AdhTAP1 infection of CMT.64 cells inhibited tumor formation in mice. *Ex vivo* infection of CMT.64 cells had a significant dose-dependent effect on both median survival time and long-term survival (Fig. 4*A*). All levels of *ex vivo* AdhTAP1 infection significantly increased median survival time compared with PBS treatment alone in a dose-dependent manner ($P < 0.01$). All mice survived the challenge with cells infected with the highest dose of AdhTAP1 (50 MOI) over the course of the experiment (100 days). Mice treated with the vector control (Ψ5) also showed significant increase in median survival time for infections at the two higher levels (10 and 50 MOI) compared with PBS treatment alone ($P < 0.01$). No significant effect was seen at the lowest level of infection (2 MOI) compared with PBS treatment ($P = 0.59$). *Ex vivo* infection of CMT.64 cells with AdhTAP1 significantly increased both median survival time and long-term survival compared with Ψ5 treatment ($P < 0.01$).

In another set of experiments, AdhTAP1 treatment of established CMT.64 tumors significantly ($P < 0.01$) increased median survival time to 68 days compared with 22 and 25 days for PBS and Ψ5 treated mice, respectively. There was also a significant effect on long-term survival. Mice treated with AdhTAP1 showed 35% long-term survival without visible tumors (>100 days) in contrast with PBS- and Ψ5-treated mice where 100% of mice died within 43 and 48 days, respectively (Fig. 4*B*). No significant increase in median survival time was observed with Ψ5-treated mice compared with PBS treatment alone ($P = 0.29$). For the *in vivo* experiments, four to eight mice from each group were examined for patterns in tumor growth 20 days after the last treatment injection. The peritoneal cavities of mice treated with AdhTAP1 were tumor-free or had only a few small tumors <1 or 2 mm in diameter. Both the liver and intestine seemed normal upon visual inspection. This was in sharp contrast with mice treated with PBS or Ψ5. These mice had large volumes of bloody ascitic fluid (2-5 mL) and countless tumors distributed throughout the peritoneal cavity. Tumors were observed growing on the liver and intestine and were associated with large fibrotic adhesions. Tumors harvested from these mice were examined for tumor-infiltrating lymphocytes and dendritic cell infiltrates by FACS and immunohistochemical staining. Immunohistochemical staining showed that mice treated with AdhTAP1 had more $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes (Fig. 5*A* and *B*) and more $CD11c^+$ dendritic cells (Fig. 5*C*) in the tumor mass than in mice treated with Ψ5 (Fig. 5*D-F*) or PBS (Fig. 5*G-I*). FACS analysis showed that mice treated with AdhTAP1 had greater tumor-infiltrating lymphocytes ($CD8^+$ = 12.4% of total cells and $CD4^+$ = 7.7% of total cells) than tumor-infiltrating lymphocytes in tumors from mice treated with Ψ5 ($CD8^+$ tumor-infiltrating lymphocytes = 2.8% of total cells and $CD4^+$ tumor-infiltrating lymphocytes = 3.4% of total cells).

Discussion

The immunohistochemical analysis of antigen-processing components in human lung carcinoma lesions indicates that APP defects are highly prevalent in human lung carcinomas. Thus, NSCLC and SCLC represent very poor targets for HLA class I–restricted cytolytic lymphocytes. Similar conclusions have been reported confirming that both lung cancer diseases are potentially poor candidates for immunotherapy by TAA immunization (25).

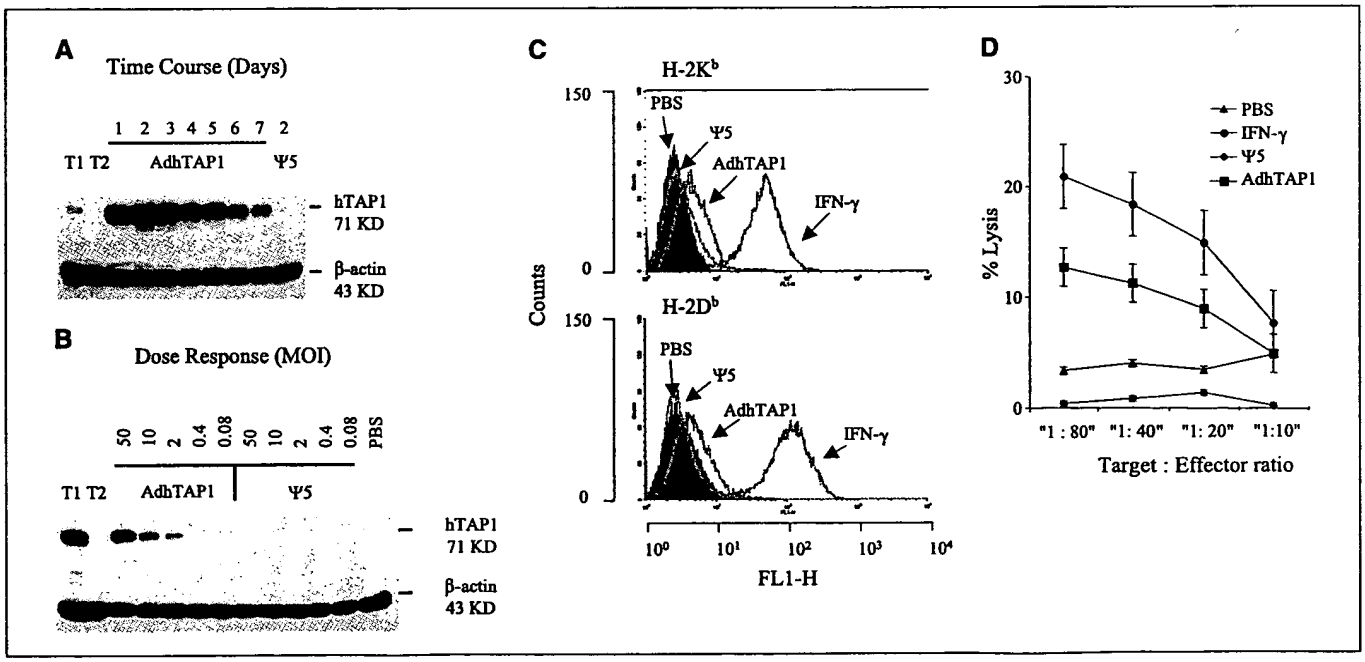

Figure 2. *A*, time course of hTAP1 expression. CMT.64 cells after infection with AdhTAP1 (MOI = 10) is visualized with a Western blot specific for hTAP1 protein. T1 cells are positive controls, T2 cells and CMT.64 cells infected with Ψ5 are adenovirus vector controls for hTAP1 expression. β-Actin was used as a control for protein loading. *B*, expression of hTAP1 is dose-dependent. CMT.64 cells were infected at MOI of 50, 10, 2, 0.4, and 0.08 with AdhTAP1 or Ψ5 and harvested 48 hours later. *C*, AdhTAP1 infection increases H-2K^b and H-2D^b surface expression in CMT.64 cells. Ψ5, adenovirus vector control; IFN-γ, positive control. *D*, infection of CMT/VSV-Np cells with AdhTAP1 restores MHC class I antigen presentation of VSV-Np epitope and increases susceptibility to lysis by VSV-Np specific effector cells. Targets: CMT/VSV-Np [CMT.64 transfected with VSV-Np (52-59) minigene] treated with PBS (mock treatment control), IFN-γ (positive control), Ψ5 (adenovirus vector control), or AdhTAP1. Effectors: splenocytes from VSV-infected mice.

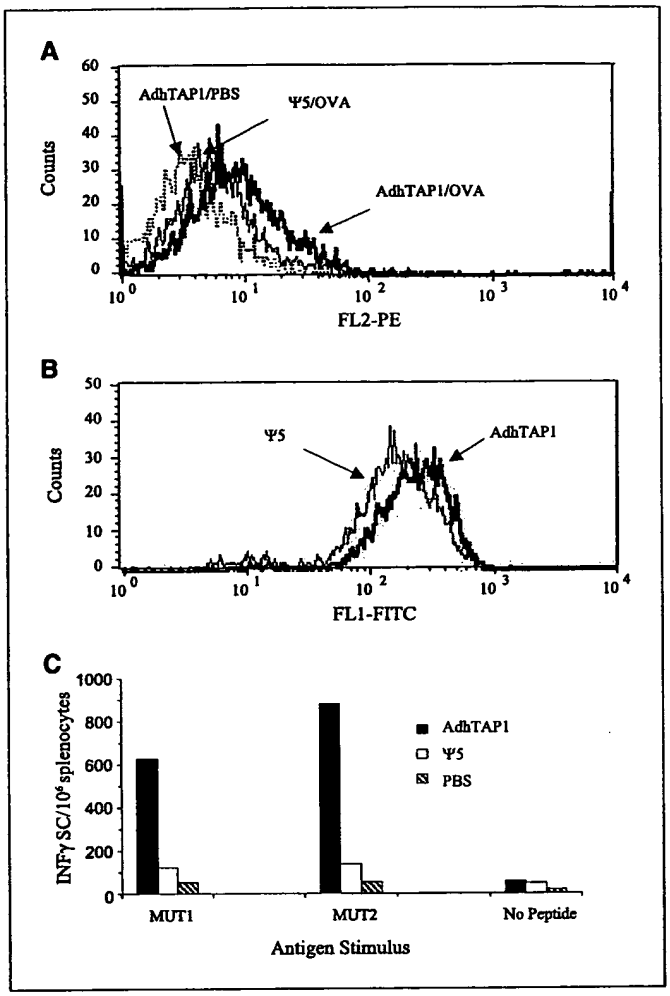

Figure 3. AdhTAP1 increases dendritic cell cross-presentation of ovalbumin antigen on MHC class I. *A*, FACS histograms of H-2K^b/ovalbumin surface complexes on dendritic cells infected with AdhTAP1 or Ψ5 after incubation with ovalbumin. AdhTAP1-infected dendritic cell incubated with PBS was a negative control for nonspecific staining of H-2K^b/ovalbumin. *B*, total H-2K^b surface complexes on dendritic cells were significantly increased by AdhTAP1 infection compared with Ψ5 infection ($P < 0.01$). *C*, immunization with irradiated CMT.64 cells infected with AdhTAP1 increased tumor-specific IFN-γ-secreting splenocytes. Bars, the mean number of IFN-γ secreting splenocytes isolated from mice immunized with γ-irradiated CMT.64 cells infected *ex vivo* with AdhTAP1, or Ψ5 (Ad vector control) or PBS (mock treatment control). Splenocytes from immunized mice were stimulated with the tumor-associated antigens, MUT1 or MUT2 or incubated without peptide. The number of tumor antigen–specific, IFN-γ-secreting precursors was determined by ELISPOT assay. Precursor frequency is reported as IFN-γ-secreting cells per 10^6 splenocytes (INF-γ SC/10^6 splenocyte).

The lack of correlation between clinical staging and APP status suggests that antigen loss variants have been established long before diagnosis and treatment of lung tumors. The results are in contrast with melanoma where significant correlations were found between APP status and disease progression (7). This may be due to earlier detection and diagnosis of primary melanomas, before antigen loss variants predominate. It is noteworthy that all patients analyzed in this study were heavy smokers (data not shown) and strengthens a recent report demonstrating that tobacco extracts inhibit TAP1 protein expression in a dose-dependent manner thereby leading to reduced HLA expression in epithelial cells (26).

In this study, the partial restoration of the APP was attempted by gene transfer in order to increase the immunogenicity of lung cancer cells. Our results show that replication-incompetent adenovirus vectors could infect murine lung carcinoma cells (CMT.64) resulting in high expression of hTAP that persists for more than 7 days. The level of expression is dependent on the dose of AdhTAP1 used to infect the cells. The persistent expression of TAP1 indicates that this high expression in itself is not toxic to the cells. The expression of the TAP1 subunit alone is capable of restoring MHC class I surface expression on the cell surface despite down-regulation of many of the components of the APP, indicating that TAP1 alone is capable of peptide transport activity (12). MHC class I appears on the cell surface after infection with AdhTAP1, but not after infection with Ψ5, indicating that this process is specific to TAP1 expression rather than an effect caused by the vector. This suggests that low but sufficient amounts of β_2-m and MHC class I heavy chain subunits are present in the endoplasmic reticulum and that these subunits assembled into MHC class I molecules when stabilized and supplied with peptides through the presence and activity of TAP1.

The level of surface expression of MHC class I, as a consequence of TAP1 expression, is capable of presenting sufficient immunogenic peptides to make the cells susceptible to antigen-specific

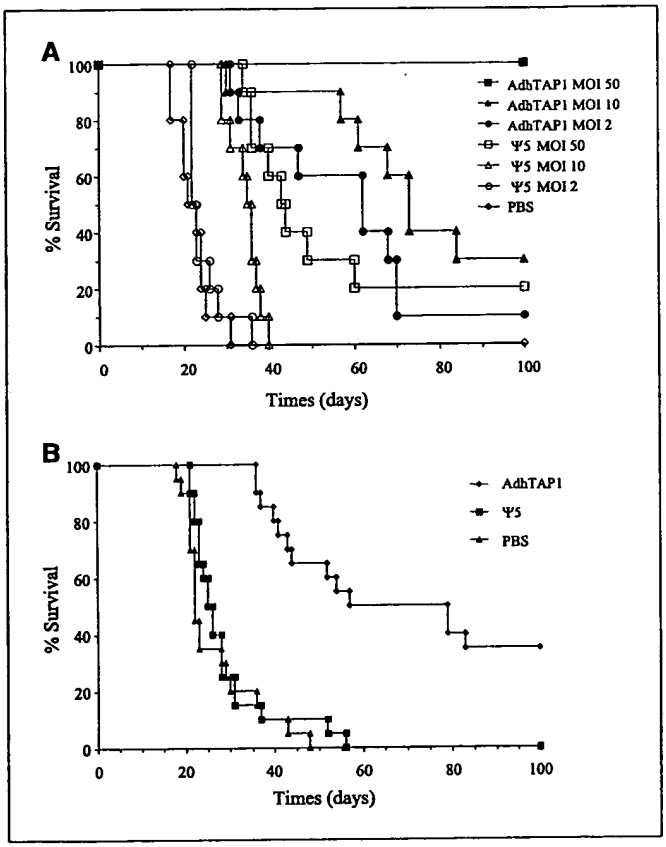

Figure 4. AdhTAP1 treatment increased survival in mice bearing CMT.64 tumors. *A*, AdhTAP1 treatment *in vitro* increased survival of CMT.64 tumor-bearing mice in a dose-dependent manner. CMT.64 cells were infected *ex vivo* with escalating dose of AdhTAP1 or Ψ5 prior to i.p. injection into mice (10 mice/treatment). Mouse survival was followed for 100 days post–tumor cell introduction. *B*, AdhTAP1 treatment *in vivo* increased survival of CMT.64 tumor-bearing mice. Three groups ($n = 20$) of mice were injected i.p. with 4×10^5 CMT.64 cells. Mice were treated with four injections i.p. of 1×10^8 PFU/mouse of AdhTAP1, Ψ5 or PBS only on day 1, 3, 5, and 8 after CMT.64 cells were introduced. AdhTAP1 provided significant survival of CMT.64-bearing mice ($P < 0.01$) compared with the Ψ5 and PBS treated mice. No difference was observed between Ψ5- and PBS-treated mice.

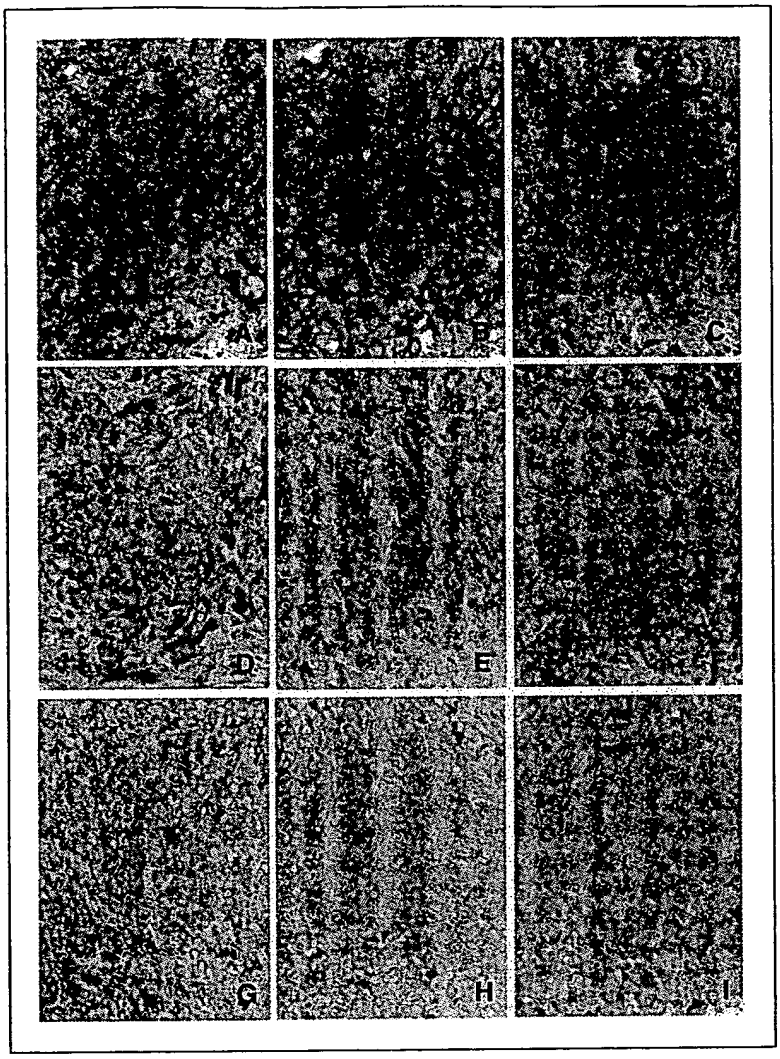

Figure 5. Immunohistochemical staining for tumor-infiltrating $CD4^+$ (*A*, *D*, and *G*) and $CD8^+$ (*B*, *E*, and *H*) lymphocytes and $CD11c^+$ dendritic cells (*C*, *F*, and *I*) in tumors treated with AdhTAP1 or Ψ5 (Ad vector control) or PBS (mock treatment control; 200× magnification).

CTL killing *in vitro*. Mice challenged with CMT.64 cells infected *ex vivo* with AdhTAP1 were able to survive the challenge provided that enough cells were infected. It is clear from the *ex vivo* animal studies that the rate of survival is dependent on viral dose. There is a major dose-dependent effect associated with the presence of TAP1 and a minor effect associated with the adenovirus vector itself. This suggests that a threshold number of cells have to express TAP1 to overcome the lack of immunologic recognition and that the viral vector alone may also stimulate the immune system to generate antitumor responses. Because CMT.64 is not known to express the costimulatory molecules required for the stimulation of a Th1-type antitumor response they would have to be processed by dendritic cells. The dendritic cells would have to acquire, process, and cross-present the tumor antigens in order to cross-prime a cellular antitumor response. The expression of hTAP1 in CMT.64 cells facilitates cross-priming, shown by the hTAP1-dependent increase in MUT1- and MUT2-specific INF-γ-secreting splenocytes. The observed increase in survival of mice receiving CMT.64 cells infected with the highest dose of Ψ5 might be due to stimulation of innate responses mediated by viral gene transcription byproducts (dsRNA) interacting with TLR 3 (27, 28). The virus vector seems to act as an adjuvant by providing a potent "danger signal" to dendritic cells and may contribute to the priming of TAP1-dependent antitumor immune responses.

The administration of AdhTAP1 to mice already bearing disseminated i.p. tumors also increases survival significantly. It is interesting and encouraging that at the doses applied in these experiments, it is remote that sizable numbers of CMT.64 cells were infected *in vivo* by AdhTAP1. It is clear, however, that the administration of AdhTAP1 stimulated an effective immune response against CMT.64 cells. This provides reinforcement that the response is due not only to increased MHC class I expression on some tumor cells but also due to some other contributing process. Presumably, this other process is at the level of cross-presentation of acquired tumor antigen to MHC class I restricted components by dendritic cells. *In vitro*, there were significant increases in the cross-presentation of exogenous ovalbumin antigens by dendritic cells infected with AdhTAP1. *In vivo*, the biological relevance of these increases observed *in vitro* are corroborated by the increase in IFN-γ-secreting TAA-specific splenocytes in mice vaccinated with irradiated AdhTAP1-infected CMT.64 cells but not Ψ5-infected cells. Further corroboration is provided by the observed increase in $CD4^+$ and $CD8^+$ T cells and $CD11c^+$ dendritic cells within tumors from AdhTAP1-treated mice.

It is intellectually satisfying that the administration of TAP immunotherapy induces the production of tumor-specific CTLs; however, it should be stressed that in addition to responses against the MUT antigens, there must also be other CTL responses to tumor-specific and tumor-associated antigens that have not yet been identified. TAP1 activity in this class of tumors would make a wide variety of antigens available for loading onto nascent MHC class I molecules. The immune response against these antigens must be sensitive to very low levels of MHC class I expression as it is unlikely that a large portion of the introduced CMT.64 cells are infected *in vivo* by AdhTAP1. In accord is the work by Purbhoo et al. (29) demonstrating that during the effector stage, CTLs were able to detect even one to three peptide-MHC complexes but required about 10 peptide-MHC complexes to achieve stable synapse formation and complete signaling to activate CTL precursors.

This study extends previous reports that have shown that the introduction of TAP1 into tumor-bearing mice increases survival. The previous study accomplished this result with replication competent vaccinia vectors (14). In this study, we show that a replication-incompetent adenovirus virus vector can also be used to establish a specific antitumor immune response against TAP-deficient CMT.64 cells. This is an important step in establishing the potential for *TAP1* gene transfer as an immunotherapy for the treatment of human cancer because these vectors are well tolerated, have a safe clinical history, and the cyclic guanosine 3′,5′-monophosphate production of these vectors is established. Overall, these studies are encouraging for the clinical implementation of recombinant adenovirus vectors encoding TAP1 and need to be extended to other types of cancer. The results further showed that TAP should be considered for inclusion in cancer therapies, as it is likely to provide a general method for increasing immune responses against tumors regardless of the antigenic complement of the tumor or the MHC haplotypes of the host.

Acknowledgments

Received 11/8/2004; revised 6/17/2005; accepted 6/29/2005.

Grant support: CIHR, CANVAC, GeneMax Corp. (to W. Jefferies); SFB 432 project A5 and Boehringer Ingelheim Stiftung (to B. Seliger).

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We thank Dr. Cheryl Pfeifer for critical reading of the manuscript.

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(Sigma), mTREM-1/IgG1, hILT-3/IgG1[16], or heat-inactivated mTREM-1/IgG1 (30 min, 95 °C) by i.p. injection at 1, 2, 4 and 6 h after or 1 h before LPS administration. We monitored viability of treated mice 4–6 times a day for at least 10 days.

Analysis of blood and peritoneal lavage

Blood (250 μl) was collected and serum TNF-α and IL-1β were determined using cytokine-specific enzyme-linked immunosorbent assays as per the manufacturer's protocol (R&D Systems). Total cell numbers of peritoneal lavage cells collected from treated and control mice were determined on a coulter counter. We performed differential counts according to standard morphological criteria on cytospin preparations stained with Giemsa/May-Grünwald solution (Sigma). Four-colour analysis of peritoneal leukocytes was performed after blocking Fc receptors (FcR blocking agent; Pharmingen) for 30 min, using anti-mTREM-1, anti-Ly-6G (Pharmingen), anti-Mac-1 (Pharmingen) monoclonal antibodies conjugated with APC, PE and FITC, respectively. Dead cells were excluded by staining with propidium iodide.

Escherichia coli peritonitis model

E. coli peritonitis was induced in mice as described[25]. Briefly, female C57BL/6 mice (8–10 weeks, 19–22 g) were weighed and randomly distributed into groups of 5–15 animals of equal body weight. Mice were injected i.p. with 500 μg of mTREM-1/IgG1 or control hIgG1 before i.p. administration of 500 μl of a suspension of *E. coli* O111:B4 ($1.6–2.1 \times 10^6$ colony forming units per mouse).

Caecal ligation and puncture

We performed CLP as described[17,24]. Briefly, female C57BL/6 mice (8–10 weeks, 19–22 g) were anaesthetized by i.p. administration of 75 mg per kg Ketanest (Parke Davies) and 16 mg per kg Rompun (Bayer AG) in 0.2 ml sterile pyrogen-free saline (B. Braun Melsungen AG). The caecum was exposed through a 1.0–1.5-cm abdominal midline incision and subjected to a 50–80% ligation of the distal half followed by a single puncture with a G23 needle. A small amount of stool was expelled from the punctures to ensure patency. The caecum was replaced into the peritoneal cavity and the abdominal incision closed in layers with 5/0 Prolene thread (Ethicon). Sterile saline (500 μl) containing 500 μg mTREM-1/IgG1, 500 μg hIgG1κ (Sigma) or 100 μg TNF-RI/IgG1 (Pharmingen) (together with 400 μg hIgG1κ; Sigma) was administered by i.p. injection immediately after CLP. The CLP was performed blinded to the identity of the treatment group. We assessed survival after CLP 4–6 times a day for at least 7 days.

Received 22 January; accepted 26 February 2001.

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Acknowledgements

We thank R. Breitkreutz and A. Benner for statistical analysis; O. Alebardi and S. Festa for technical assistance in immunohistochemistry; N. Schmitz for advice during mouse experiments; M. Cella, R. Ettinger, F. McBlane, M. Kopf, F. Sinigaglia and R. Torres for reviewing the manuscript; and P. Krammer for discussion. This work was partly supported by a BIOMED-2 grant to F.F. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche, CH-4002 Basel.

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IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity

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Lymphocytes were originally thought to form the basis of a 'cancer immunosurveillance' process that protects immunocompetent hosts against primary tumour development[1,2], but this idea was largely abandoned when no differences in primary tumour development were found between athymic nude mice and syngeneic wild-type mice[3–5]. However, subsequent observations that nude mice do not completely lack functional T cells[6,7] and that two components of the immune system—IFNγ[8,9] and perforin[10–12]—help to prevent tumour formation in mice have led to renewed interest in a tumour-suppressor role for the immune response. Here we show that lymphocytes and IFNγ collaborate to protect against development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas and also to select for tumour cells with reduced immunogenicity. The immune response thus functions as an effective extrinsic tumour-suppressor system. However, this process also leads to the immunoselection of tumour cells that are more capable of surviving in an immunocompetent host, which explains the apparent paradox of tumour formation in immunologically intact individuals.

Age-matched female wild-type mice and immunodeficient mice with a targeted disruption of the recombination-activating gene-2 (RAG2) that is expressed only in lymphocytes[13], both on a pure 129/SvEv genetic background, were injected subcutaneously with 100 μg of the chemical carcinogen methylcholanthrene (MCA) and monitored for tumour development. $RAG2^{-/-}$ mice developed tumours earlier than wild-type mice and with greater frequency ($P < 0.01$). After 160 days, 9/15 $RAG2^{-/-}$ mice but only 2/15 wild-type mice formed MCA-induced tumours (Fig. 1a). Similar results

were obtained in two additional experiments. In total, 30/52 RAG2$^{-/-}$ mice formed MCA-induced tumours compared to only 11/57 wild-type mice (Fig. 1b) ($P < 0.0001$). The increased tumour formation observed in RAG2$^{-/-}$ mice was comparable to that observed in 129/SvEv strain, IFNγ-insensitive mice that lack either the IFNγ receptor (IFNGR1) or STAT1, the transcription factor that plays a dedicated role in mediating signalling by the IFNγ and IFNα/β receptors[14,15] (12/20 and 17/30, respectively, versus 11/57 wild-type mice; $P < 0.001$); this data is consistent with our previous findings[9] (Fig. 1b). To assess the extent of collaboration between the lymphocyte- and IFNγ/STAT1-dependent tumour-suppressor mechanisms, we generated RAG2$^{-/-}$ × STAT1$^{-/-}$ mice (RkSk mice) and assessed their MCA sensitivity. Mice lacking both genes also showed increased susceptibility to MCA-induced carcinogenesis with 13/18 mice forming tumours compared to 11/57 wild-type mice ($P < 0.0001$), but did not display a significantly greater tumour incidence compared to mice that lacked either the RAG2 or STAT1 genes individually (Fig. 1b) ($P = 0.127$ and 0.140, respectively). All four groups of mice formed histologically indistinguishable sarcomas at the injection site. Similar results were obtained using male mice, which are known to be more susceptible to MCA-induced tumorigenesis. Thus, T, NKT and/or B cells are essential to suppress development of chemically induced tumours.

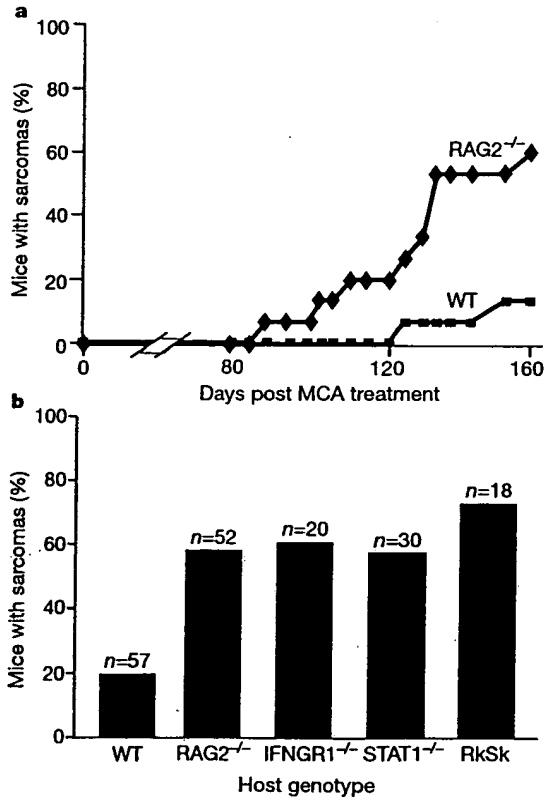

Figure 1 Lymphocyte-deficient mice are highly susceptible to MCA-induced tumour development. **a**, Fifteen female RAG2$^{-/-}$ mice (diamonds) and 15 syngeneic 129/SvEv wild-type (WT) mice (squares) were injected with a single subcutaneous dose of 100 μg MCA and observed for tumour development for 160 days. This dose and observation period were determined to be the most informative based on previous carcinogenesis experiments performed on the 129/SvEv strain of mice[9]. Data are represented as a percentage of each group of mice bearing tumours at least 9 mm in diameter as a function of time. **b**, Cumulative tumour formation at 160 days from three independent experiments involving female wild-type 129/SvEv mice ($n = 57$), RAG2$^{-/-}$ mice ($n = 52$), STAT1$^{-/-}$ mice ($n = 30$), IFNGR1$^{-/-}$ mice ($n = 20$) and RAG2$^{-/-}$ × STAT1$^{-/-}$ (RkSk) mice ($n = 18$) after injection of 100 μg MCA.

Given the comparable effects on tumour development of eliminating lymphocytes or STAT1-dependent IFNγ signalling, either individually or together, there is an extensive overlap between the two tumour-suppressor systems.

To determine whether these tumour-suppressor systems also function to prevent the formation of spontaneous tumours, we monitored tumour development in unmanipulated 129/SvEv wild-type mice, RAG2$^{-/-}$ mice and RkSk mice. As neither wild-type nor RAG2$^{-/-}$ mice showed outward signs of disease during the observation period, animals were killed and evaluated for neoplasia upon reaching a minimum age of 15 months. At necropsy, 9/11 wild-type mice (15–21 months) were free of neoplastic disease, one (16.1 months) developed an intestinal adenoma and one (19 months) displayed a harderian gland cystadenoma, but none had cancer (Fig. 2a and Supplementary Information). In contrast, 12/12 RAG2$^{-/-}$ mice of ages 15–16 months displayed neoplastic lesions in the intestinal tract and elsewhere (Fig. 2b and Supplementary Information). Half of these mice developed malignant neoplasias (three had caecal adenocarcinomas, one had an adenocarcinoma at the ileocaecal junction, one had an adenocarcinoma of the small intestine and one had a lung adenocarcinoma) that grew progressively when transplanted into naive RAG2$^{-/-}$ mice. Six of eleven RkSk mice developed overt mammary-gland carcinomas (one had two mammary adenocarcinomas and another carried distinct adenocarcinomas in the breast and caecum) that were detected well before the mice reached 15 months (Fig. 2c and Supplementary Information). This result is particularly striking given the extremely low frequency of spontaneous mammary tumours in wild-type mice or RAG2$^{-/-}$ mice of 129 origin (Fig. 2a, b) and the very late appearance (>20

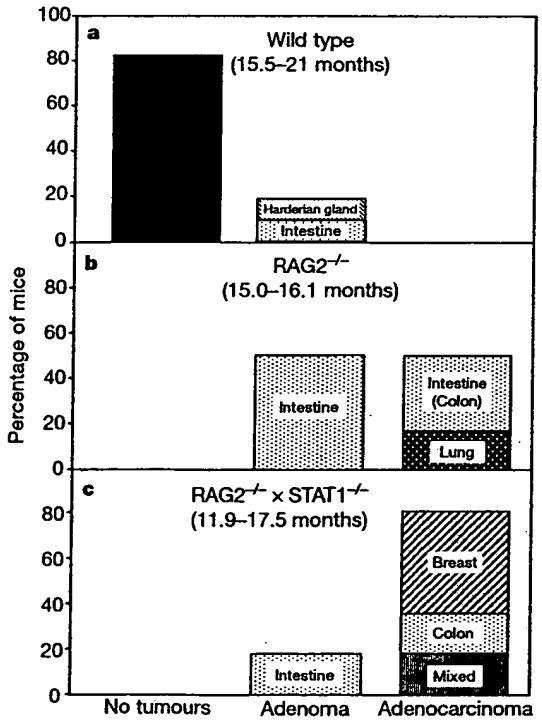

Figure 2 Increased development of spontaneous neoplastic disease in immunodeficient mice. Eleven wild-type 129/SvEv mice (**a**), 12 RAG2$^{-/-}$ mice (**b**) and 11 RkSk mice (**c**) were housed in a room that was free of specific pathogens including *Helicobacter*. Mice were killed when they appeared morbid, developed overt masses, or upon reaching an age of 15–21 months. Organs were examined for gross pathology, and histologic sections were analysed microscopically by three independent pathologists. Additional details of tumour formation and histologic sections from representative mice can be viewed as Supplementary Information.

months) of mammary tumours in mice that lack only the STAT1 gene (data not shown). Cells from mammary carcinomas grew progressively when transplanted into naive RkSk mice. The other five RkSk mice did not display palpable masses but at necropsy two had adenocarcinomas in the caecum, one had adenocarcinomas in the caecum and lung and the other two displayed intestinal adenomas. In total, 82% of the RkSk group developed spontaneous cancer. Thus, mice that lack lymphocytes, either alone or in combination with an IFNγ signalling deficit, are significantly more prone to spontaneous epithelial tumour development than their wild-type counterparts ($P < 0.01$ and 0.001, respectively). Moreover, because RkSk mice develop more spontaneous cancers than RAG2$^{-/-}$ mice ($P < 0.01$), the tumour-suppressor mechanisms mediated by lymphocytes and IFNγ/STAT1 must only partially overlap.

To assess whether the immune system influences the immunogenic phenotype of tumours formed during chemical carcinogenesis, the tumorigenicities of MCA-induced sarcomas from RAG2$^{-/-}$ and wild-type mice were compared using tumour transplantation approaches. When injected into RAG2$^{-/-}$ mice, both wild-type- and RAG2$^{-/-}$-derived sarcomas grew progressively with equivalent kinetics (Fig. 3a, b). When transplanted into naive syngeneic immunocompetent hosts, 17/17 different tumours from wild-type mice grew progressively (Fig. 3c). In contrast, 8/20 (40%) distinct tumours from MCA-treated RAG2$^{-/-}$ mice were rejected following transplantation into immunocompetent mice even when injected at high tumour cell inocula (10^6 cells per mouse) (Fig. 3d). The rejection of tumours derived from the immunodeficient hosts was not due to potential minor genetic differences between wild-type 129/SvEv and RAG2$^{-/-}$ 129/SvEv mice, as the tumours were rejected when transplanted into wild-type × RAG2$^{-/-}$ F1 mice. Moreover, we ruled out the possibility that the rejection of the RAG2$^{-/-}$ tumours was due to immune responses against retrovirus-related antigens as the tumours (1) did not produce infectious amphotropic or ecotropic retrovirus (plaque assays), (2) did not express retroviral gp70 messenger RNA (RT-PCR) and (3) did not express MuLV-related antigens (flow cytometry using G_{IX} MuLV antiserum[16]) (data not shown). Thus, tumours that arise in lymphocyte-deficient mice are more immunogenic than those that develop in the presence of an intact immune system. Taken together with our previous findings that IFNγ-unresponsive mice develop more tumours than wild-type mice and that IFNγ responsiveness of the tumour cell is essential for effective immune recognition[9], these results show that lymphocytes and the IFNγ/STAT1 signalling pathway collaborate with one another to shape the immunogenic phenotype of tumours that eventually form in immunocompetent hosts.

To determine the link between the lymphocyte-dependent and IFNγ/STAT1-dependent tumour-suppressor processes, we asked whether the highly tumorigenic phenotype of IFNγ-insensitive tumour cells could be eliminated by selectively expressing in them components of the MHC class I processing and presentation pathway that are known to be significantly upregulated by IFNγ. We studied two pathway components, TAP1 and the H-2K^b heavy chain, because the decreased or absent expression of these proteins is a mechanism that tumours use to escape immune detection[17]. This mechanism is also used by tumours that develop inactivating mutations in the genes that encode the proximal IFNγ receptor signalling proteins (ref. 9; V.S. & R.D.S., unpublished work). Our focus on TAP1 was also prompted by a previous report showing that expression of rat TAP1 in a mouse small-cell lung-carcinoma cell line decreased its tumorigenicity[18], although this study was complicated by the use of xenogeneic TAP1, which differs from the endogenous mouse protein at 80 positions[19].

We chose to use for these studies two distinct IFNγ-insensitive sarcomas (RAD.gR.28 and RAD.gR.30) derived from IFNGR1$^{-/-}$ 129/SvEv mice because we had (1) established previously that

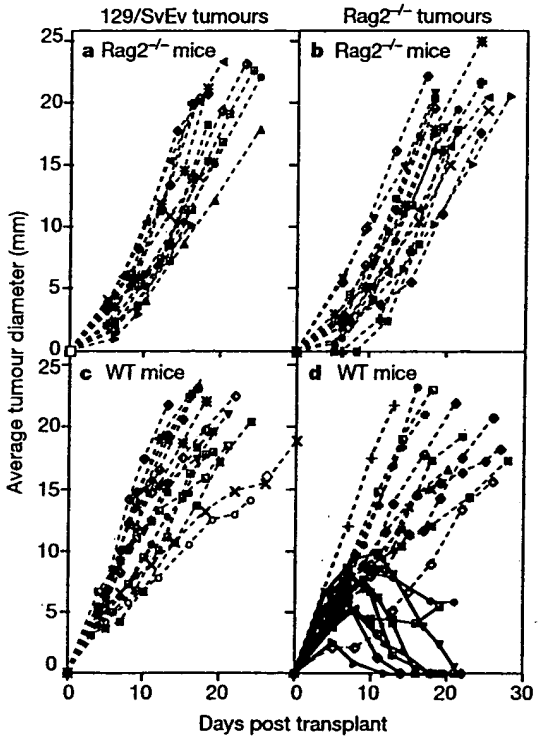

Figure 3 Increased immunogenicity of tumours derived from MCA-treated RAG2$^{-/-}$ mice. Immunodeficient RAG2$^{-/-}$ hosts were inoculated on day 0 with a dose of 10^5 tumour cells derived from wild-type 129/SvEv mice (a) or RAG2$^{-/-}$ mice (b). Tumour growth is plotted as mean tumour diameter of 3–5 mice inoculated with a distinct tumour. Groups of 5–8 immunocompetent 129/SvEv × RAG2$^{-/-}$ F1 mice were inoculated on day 0 with doses of 10^6 tumour cells derived from 17 individual 129/SvEv mice (c) or 20 individual RAG2$^{-/-}$ mice (d) and tumour growth monitored as above. In d, the dashed lines denote tumours that grew progressively, whereas solid lines represent tumours that were rejected.

sarcomas lacking either the IFNGR1 subunit or STAT1 display common biologic response deficits and enhanced *in vivo* growth and (2) defined, in great detail, the *in vivo* growth behaviour of IFNGR1$^{-/-}$ sarcomas before and after reconstitution of IFNγ sensitivity[9]. Both tumour cell lines, which express low but detectable amounts of TAP1 and H-2K^b protein (H-2K^b), were stably transfected with expression plasmids encoding the 129/SvEv haplotypes of TAP1 or H-2K^b, and clones were selected that expressed high protein levels comparable to those expressed in IFNγ-treated, IFNγ-responsive cells (see Supplementary Information). Parental RAD.gR28 cells (Fig. 4a), empty vector-transfected RAD.gR28.neo cells (Fig. 4b) and 2/2 clones of H-2K^b-transfected RAD.gR28.K^b cells (Fig. 4a) grew progressively in immunocompetent mice when injected at 10^6 cells per mouse. In contrast, TAP1-transfected RAD.gR28.TAP1 cells formed small subcutaneous masses that expanded for the first 5–10 days but then disappeared two weeks after inoculation (Fig. 4b). This effect was generalizable to 8/8 independent RAD.gR28.TAP1 clones (two representative clones are shown in Fig. 4b). In addition, the same result was obtained with 2/2 clones of a second IFNγ-insensitive tumour cell line (RAD.gR30) engineered for TAP overexpression (Fig. 4c). Rejection of TAP1-transfected IFNγ-insensitive tumour cells required the participation of both CD4$^+$ and CD8$^+$ T cells because rejection of the TAP1-reconstituted tumour cells occurred only in wild-type mice and not in lymphocyte-deficient RAG2$^{-/-}$ mice (Fig. 4d) and was abrogated in wild-type recipients depleted of either CD4$^+$ or CD8$^+$ T cells (Fig. 4e). We found that wild-type mice that rejected the TAP1-reconstituted IFNγ-insensitive tumour were immune to subsequent challenge with either the same reconstituted tumour cell line or the unreconstituted IFNγ-insensitive parental tumour cell line (Fig. 4f), a result that supports the concept that higher antigen expression is needed for the induction of an immune response to a tumour than is required for recognition of a tumour by a pre-sensitized host. Taken together, these results reveal that TAP1 represents at least one link between tumour suppression manifested by the IFNγ signalling pathway and lymphocytes.

In this study, we show that the immune response is essential to prevent the development of carcinogen-induced sarcomas and spontaneous epithelial tumours. We also show that the tumour-suppressor function of the immune system is critically dependent on the actions of IFNγ, which, at least in part, are directed at regulating tumour-cell immunogenicity. These studies thus provide experimental evidence that, in principle, supports the original concept of cancer immunosurveillance. However, as tumours that develop in the presence of an intact immune system are less immunogenic than those that develop in immunodeficient hosts, the actions of the immune system also paradoxically favour the eventual outgrowth of tumours that are more capable of escaping immune detection. Thus, tumours are imprinted by the immunologic environment in which they form. For this reason, 'cancer immunosurveillance' is not the best term to describe the process because the concept, in its original form, implied that the immune system is involved only at the initial stages of cellular transformation and plays a purely protective role[1,2]. Rather, we favour the use of the term 'cancer immunoediting' to describe better the protective and sculpting actions of the immune response on developing tumours that probably occur continuously during tumour development. We envisage the scope of this process to be very broad, with a potential

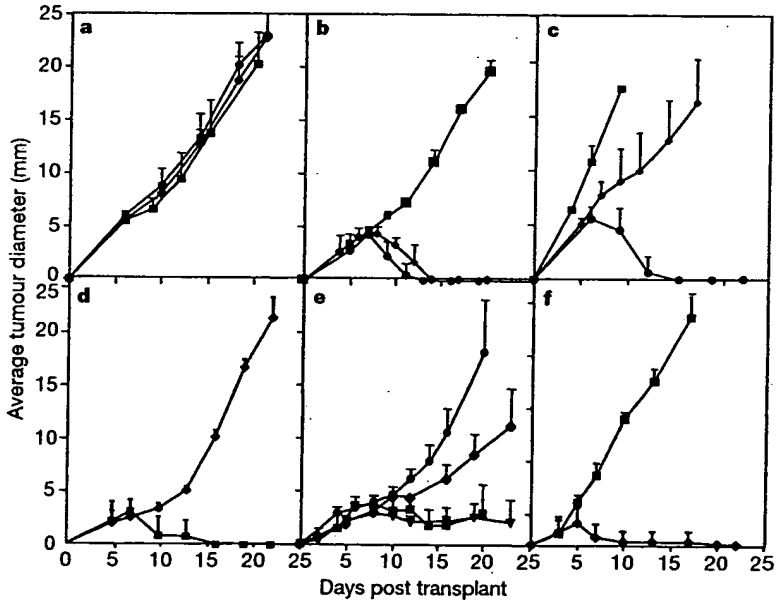

Figure 4 T-lymphocyte-dependent rejection of RAD.gR28.TAP1 cells by immunocompetent hosts. **a**, Wild-type mice were injected subcutaneously on day 0 with inocula of 10^6 RAD.gR28 (squares) cells or two representative H-2K^b-transfected RAD.gR28 clones denoted RAD.gR28.K^b.15 (diamonds) or RAD.gR28.K^b.30 (circles). **b**, One million mock-transfected RAD.gR28.neo cells (squares) or the same number of two representative TAP1-transfected RAD.gR28 clones denoted RAD.gR28.TAP1.8 (diamonds) or RAD.gR28.TAP1.16 (circles), were injected into syngeneic immunocompetent animals. **c**, 129/SvEv mice were injected with 10^6RAD.gR30 (squares), RAD.gR30.neo (diamonds) or RAD.gR30.TAP1 (circles) cells. Tumour diameter in the syngeneic hosts was monitored over time. **d**, RAD.gR28.TAP1 cells (10^6 cells per mouse) were injected into the flanks of wild-type mice (squares) or RAG2$^{-/-}$ mice (diamonds) and monitored for tumour growth. **e**, In a separate experiment RAD.gR28.TAP1 growth (10^6 cells per mouse) was compared after injection into wild-type 129/SvEv mice (squares) and wild-type mice depleted of CD8$^+$ T cells (circles) or wild-type mice (triangles) and wild-type mice depleted of CD4$^+$ T cells (diamonds). CD8$^+$ T cells were depleted by injecting mice intraperitoneally with 100 μg of purified YTS-169.4 anti-CD8 mAb seven days before tumour transplantation and once weekly thereafter. CD4$^+$ T cells were depleted by injection of 250 μg GK1.5 anti-CD4 mAb two days before tumour transplantation and every other day thereafter. Depletion of each T-cell subset was monitored by flow cytometry analysis on peripheral blood using FITC-labelled CD4 and CD8-specific mAbs directed against epitopes that were distinct from those recognized by the depleting antibodies. **f**, Growth of 10^6 parental RAD.gR28 tumour cells was assessed in naive 129/SvEv animals (squares) or 129/SvEv mice that had rejected on inoculum of 10^6 live RAD.gR28.TAP1 cells three weeks earlier (diamonds). Data are represented as average tumour diameter ± s.d. of 4–6 mice per group as a function of time.

to (1) promote complete elimination of some tumours, (2) generate a non-protective immune state to others, or (3) favour the development of immunologic anergy/tolerance/indifference. Future work is needed to define the molecular basis of the cancer immunoediting process. □

Methods

Mice

$RAG2^{-/-}$, $IFNGR1^{-/-}$ (ref. 20) and wild-type mice, all on a 129/SvEv background, were either purchased from Taconic Farms or bred in our specific pathogen-free animal facility. $STAT1^{-/-}$ mice, generated in our laboratory, were maintained on a pure 129/SvEv background[14]. For the generation of $RAG2^{-/-} \times STAT1^{-/-}$ mice, the status of the RAG2 locus was followed by assaying blood for $CD3^+$ cells by FACS as previously described[21]. Genotyping of the STAT1 locus was performed by polymerase chain reaction as previously described[9].

MCA tumour induction and transplantation

Performed as previously described[9]. A new preparation of 3'-methylcholanthrene dissolved in peanut oil was used in each experiment. Progressively growing masses 9 mm and larger were scored as tumours and confirmed by histology. The threshold value of 9 mm was chosen because masses of this size invariably continued to increase in size. To eliminate the possibility that the rejection of tumours derived from $RAG2^{-/-}$ mice was due to minor antigenic differences between $RAG2^{-/-}$ mice and 129/SvEv mice, $RAG2^{-/-}$ or Taconic 129/SvEv tumours were transplanted into immunocompetent mice that were generated by mating male 129/SvEv mice (purchased from Taconic) to female $RAG2^{-/-}$ mice.

Determination of retrovirus expression in tumour cells

The feline S^+L^- focus assay (which tests for live amphotropic retrovirus) and the XC plaque assay (which tests for live B- and N-ecotropic murine retrovirus) were performed on supernatants from 12 different $RAG2^{-/-}$ tumour cultures by BioReliance. Eighteen different sarcomas from $RAG2^{-/-}$ mice and eleven different sarcomas derived from 129/SvEv wild-type animals were tested for reactivity to the G_{IX} rat antisera that reacts with several murine leukaemia virus (MuLV) proteins[16]. Primers within conserved regions of the gp70 gene were used to amplify a 335-base-pair band from the reverse-transcribed RNA of 16 different $RAG2^{-/-}$ tumours and 7 different wild-type tumours.

Received 12 January; accepted 28 February 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank J. J. Monaco for providing a cDNA encoding TAP1 and T. H. Hansen for providing TAP1 antibodies and the cDNA encoding H-$2K^b$. We thank M. La Regina for performing the veterinary pathology and C. Hughley and C. Arthur for expert technical assistance. We also wish to thank P. M. Allen, K. M. Murphy, A. S. Shaw and E. R. Unanue for helpful suggestions during the preparation of this manuscript and J. F. Piccirillo for assistance with the statistical analysis of the data. This work was supported by grants from the NIH and the Cancer Research Institute. V.S. was supported and A.T.B. is supported by a pre-doctoral training grant from the Cancer Research Institute (CRI) and H.I. is supported by the John Hans and Edna Alice Old Post-Doctoral Fellowship from the CRI.

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Somatic activation of the *K-ras* oncogene causes early onset lung cancer in mice

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About 30% of human tumours carry *ras* gene mutations[1,2]. Of the three genes in this family (composed of *K-ras*, *N-ras* and *H-ras*), *K-ras* is the most frequently mutated member in human tumours, including adenocarcinomas of the pancreas (~70–90% incidence), colon (~50%) and lung (~25–50%)[1–6]. To constuct mouse tumour models involving *K-ras*, we used a new gene targeting procedure to create mouse strains carrying oncogenic alleles of *K-ras* that can be activated only on a spontaneous recombination event in the whole animal. Here we show that mice carrying these mutations were highly predisposed to a range of tumour types, predominantly early onset lung cancer. This model was further characterized by examining the effects of germline mutations in the tumour suppressor gene *p53*, which is known to be mutated along with *K-ras* in human tumours. This approach has several advantages over traditional transgenic strategies, including that it more closely recapitulates spontaneous oncogene activation as seen in human cancers.

The effects of *ras* gene mutations have been studied in transgenic mice; however, most strains expressed *H-ras* or *N-ras* (reviewed in ref. 7). Traditional transgenic strategies direct expression of the oncogene in all cells of the target tissue and may lead to supraphysiological levels of expression. In an effort to construct a *ras*-based mouse tumour model that overcomes these limitations, we have used a variation of 'hit-and-run' gene targeting[8] to create new mouse strains harbouring latent, oncogenic alleles of *K-ras* capable of spontaneous activation *in vivo*.

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